REMARKS

Upon entry of the foregoing amendments, claims 21-23, 29-31 and 34-36 are under consideration. Claims 26, 28, 32, 33, 37, and 38 were canceled herein, without prejudice or disclaimer. Applicant reserves the right to prosecute the cancelled subject matter, as well as the originally presented claims, in continuing applications. No new matter is added.

§ 112, First Paragraph Rejection: Written Description

The Examiner has rejected claims 21-23, 26 and 28-38 under 35 U.S.C. § 112 first paragraph for lack of written description. This is a new matter rejection. The Examiner asserts that the specification and the claims as originally filed does not provide support for the invention as now claimed. Claim 26, 28, 32, 33, 37, and 38 have been canceled. The remaining rejections are addressed as follows.

A. "an immunoglobulin heavy chain polypeptide"

Regarding claims 21, 26, and 29, the Examiner states that the specification and the claims as originally filed do not provide support for the phrase "an immunoglobulin heavy chain polypeptide." Applicants have amended claims 21, 26, and 29 to delete the phrase "an immunoglobulin heavy chain polypeptide" and to recite "an immunoglobulin Fc region". Applicants traverse this rejection to the extent it applies to the claims as amended.

The phrase "immunoglobulin Fc region" is supported by the specification as filed. Specifically, the specification recites at page 8, lines 6-10, "[t]he mucin/immunoglobulin expression plasmid was constructed by fusing the PCT-amplified cDNA of the extracellular part of PSGL-1 in frame via a BamHI site, to the *Fc part* (hinge, CH2 and CH3) of mouse IgG_{2b} carried as an expression cassette in CDM7" (emphasis added).

Furthermore, the specification recites "the antigenic fusion proteins according to the invention further comprises a part which confers immunoglobulin properties...the part that confers immunoglobulin properties is as an immunoglobulin or part there of...preferably said part that confers immunoglobulin properties is the *Fc part of an immunoglobulin molecule.* (*See specification*, at page 5 line 27 through page 6 line 2, emphasis added) Accordingly, as the specification clearly supports the specific part of the immunoglobulin molecule as recited in

amended claims 21, 26, and 29, it would be clear to one skilled in the art that that applicants had possession of the claimed invention. The written description rejection should be withdrawn.

B. "the extracellular portion of a P-selectin glycoprotein ligand-1"

Regarding claim 29, The Examiner states that the specification and the claims as originally filed do not provide support for the phrase "extracellular portion of a P-selectin glycoprotein ligand-1." (Office Action, page 4) Applicants traverse.

First, the specific portion of PSGL-1 claimed –the extracellular portion of PSGL-1-- is clearly supported by the as filed specification. See page 8, lines 6-8, which recites that "[t]he mucin/immunoglobulin expression plasmid was constructed by fusing the PCR-amplified cDNA of the extracellular part of PSGL-1 in frame via a BamHI site." (Emphasis added). For this reason alone – i.e., the claim being literally supported by the specification -- a rejection based on lack of written description is not proper.

Second, claim 29 clearly recites a specific piece of a PSGL-1 molecule – the extracellular portion (selected as a portion of the full length PSGL-1 integral membrane protein containing an (1) extracellular region, (2) a transmembrane region and (3) an intracellular region). To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can *reasonably* conclude that the inventor had possession of the claimed invention. Here the ordinarily skilled artisan would clearly conclude that Applicants had possession of a fusion polypeptide comprising the extracellular part of PSGL-l, when in fact the claimed construct is the construct the Applicants exemplified in the specification.

The written description rejection should be withdrawn.

C. "more Galα1, 3Gal epitopes than a wild-type P-selectin glycoprotein ligand-1"

Regarding claims 28, 33 and 38, the Examiner states that the specification and the claims as originally filed do not provide support for the phrase "comprises more Galα1, 3Gal epitopes

than the human wild-type P-selectin glycoprotein ligand-1." Claim 28, 33 and 38 have been canceled, thus this rejection if moot and should be withdrawn.

D. "comprises a part of a P-selectin glycoprotein ligand-1 that mediates binding to selectin"

Regarding claim 34, the Examiner states that the specification and the claims as originally filed do not provide support for the phrase "comprises a part of a P-selectin glycoprotein ligand-1 that mediates binding to selectin." Specifically, the Examiner asserts that the specification discloses "an essential part" of PSGL-1 but not "the part that mediates binding to selectin".

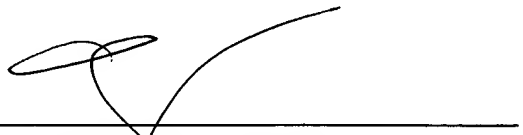
Written description is satisfied through a disclosure or relevant identifying characteristics. What is well known in the art need not be disclosed. The subject matter need not be described literally in order to be sufficient all that is required is that the specification convey clearly to those skilled in the are in any way the information that the applicant has invented the specific subject matter claimed. (In Re Lukach, 442 F.2d 967,968, 169 USPQ 795 (CCPA 1971). At the time of filing of the instant application is was well known that the primary function of PSGL-1 is as a counter receptor for P-selection -- that is PSGL-1 binds P-selectin (B. Seed, Cell (1995) 83:333-343. (Exhibit 1; "Seed"). Specifically, the ordinarily skilled artisan at the time of filing of the instant application would have known that the amino terminus of PSGL-1 is required for selectin binding. In fact, Seed teaches that a short segment near the amino terminus, specifically the first 100 amino acids, is *essential for P-selectin binding*. In addition, D.Sako et al Cell (1995) 83:323-331 (Exhibit 2) further teaches that the first 19 amino acids of the PSGL-1 polypeptide are capable of binding P-selectin. In short, one of ordinary skill in the art would know that the "essential part" of PSGL-1 is the part that is responsible for binding selectin.

For this reason, Applicants believe that it would be clear to one ordinarily skilled in the art that that applicants had possession of the claimed invention. Applicants request that this rejection be withdrawn.

CONCLUSION

Applicants believe that the claims, as amended, are in condition for allowance. If the Examiner has any questions, the Examiner is invited to contact the undersigned by telephone.

Respectfully submitted,



Dated: October 18, 2004

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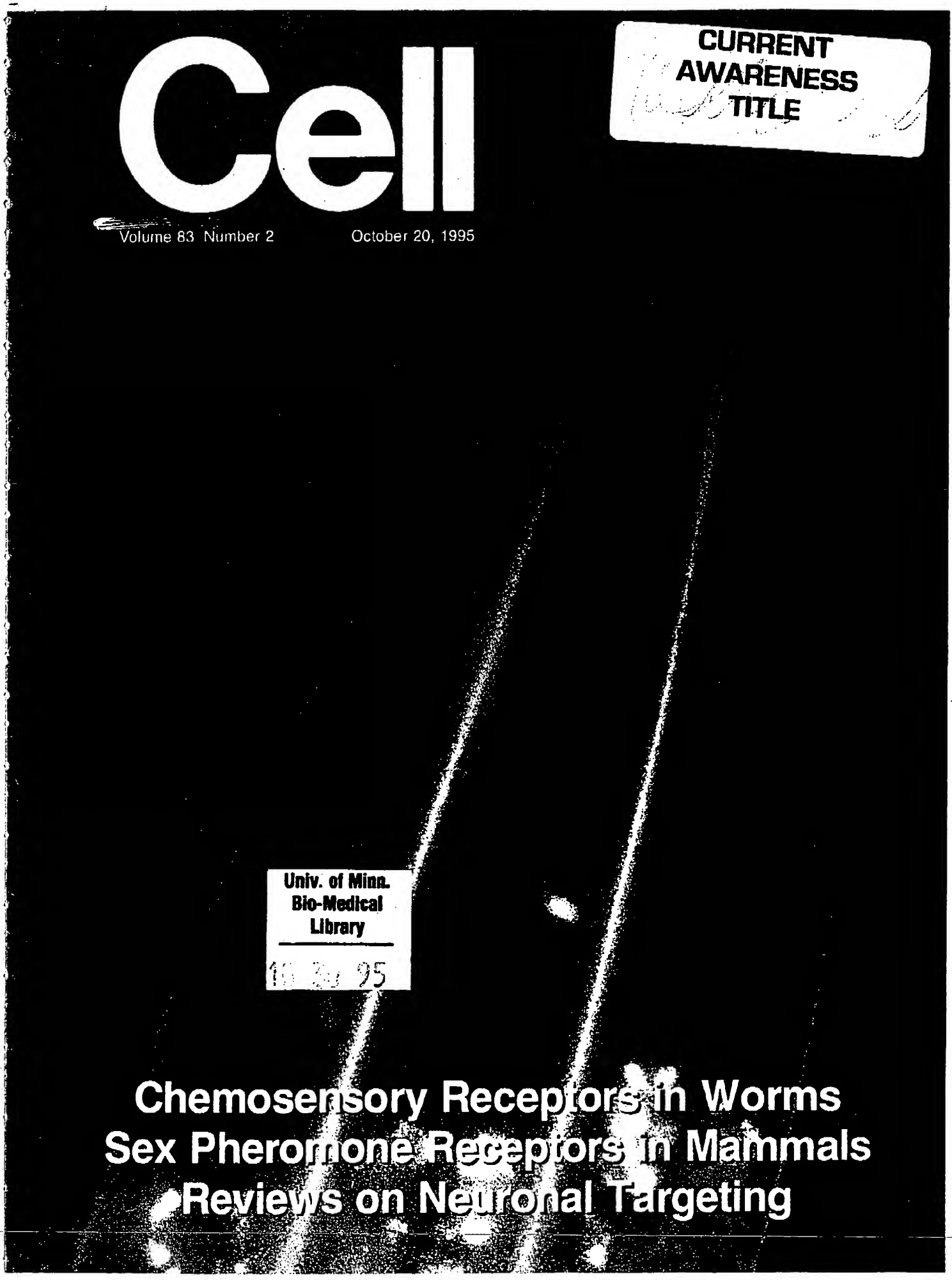

Chemosensory Receptors in Worms
Sex Pheromone Receptors in Mammals
Reviews on Neuronal Targeting

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PSGL-1 Recognition of P-Selectin Is Controlled by a Tyrosine Sulfation Consensus at the PSGL-1 Amino Terminus

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Summary

P-selectin binding to neutrophils requires a specific protein, P-selectin glycoprotein ligand 1 (PSGL-1), as well as sialyl-Lewis X (sLe^x) glycan determinants. We have found that a short segment near the amino terminus of PSGL-1 that contains a tyrosine sulfation consensus is essential for P-selectin adhesion and that addition of the amino-terminal segment to some but not all mucin-like molecules confers on those molecules the ability to bind P-selectin. PSGL-1 synthesized in the presence of sulfation inhibitors binds P-selectin weakly, and within the amino-terminal 20 residues, mutation of the tyrosines to phenylalanine abolishes binding. Rolling of HL-60 cells on P-selectin-coated coverslips is strongly attenuated by treatment of cells with an inhibitor of sulfation.

Introduction

P-selectin is an integral membrane C-type lectin found within the Weibel–Palade bodies of endothelial cells (McEver et al., 1989; Bonfanti et al., 1989) and the α granules of platelets (Hsu-Lin et al., 1984; Stenberg et al., 1985). Its translocation to the plasma membrane can be induced by thrombin (Hsu-Lin et al., 1984; Stenberg et al., 1985), histamine, and other mediators released by mast cell activation (Hattori et al., 1989a; Kubes and Kanwar, 1994; Thorlacius et al., 1994), complement C5b-9 complex or C5a fragment (Hattori et al., 1989b; Foreman et al., 1994), and peroxides (Patel et al., 1991). Once displayed on the cell surface, P-selectin supports the attachment of myelomonocytes to platelets (Larsen et al., 1989; Hamburger and McEver, 1990) or endothelial cells (Geng et al., 1990; Gamble et al., 1990). In the latter setting, its appearance heralds an underlying tissue insult and supports the initial step in leukocyte extravasation, the rolling of neutrophils along the postcapillary venule wall (Lawrence and Springer, 1991). Mice homozygously deficient for the P-selectin structural gene exhibit deficits in leukocyte rolling and show delayed recruitment of granulocytes to sites of experimentally induced inflammation (Mayadas et al., 1993).

If there is a unifying theme to the pattern of mediators that induce P-selectin expression, it is the integration of signals that commonly arise in the context of trauma or wounding. One of the first recognized responses to tissue trauma is mast cell activation, which is accompanied by release of histamine, serotonin, and other diffusible mediators. Although induction of P-selectin-mediated neutrophil rolling has been thought to be an inevitable consequence of surgical intervention, cromolyn, an agent that blocks mast cell degranulation, has been shown to prevent such rolling, thereby providing an elegant demonstration of the role of the mast cell as the link between trauma and extravasation (Kubes and Kanwar, 1994).

Much is presently known about the residues on P-selectin that are important for binding (Hollenbaugh et al., 1993; Erbe et al., 1993; Bajorath et al., 1994; Kansas et al., 1994; Murphy and McGregor, 1994), but the precise structure on human neutrophils that serves as a ligand has proven elusive. Soluble human P-selectin chimeras recognize a specific homodimeric counterreceptor that bears sialylated, fucosylated O-linked glycans (Moore et al., 1992, 1994; Norgard et al., 1993). A cDNA encoding this counterreceptor, termed P-selectin glycoprotein ligand 1 (PSGL-1) (Sako et al., 1993), was isolated by expression cloning in COS cells cotransfected with a fucosyltransferase, FTIII (Kukowska-Latallo et al., 1990), known to give rise to Lewis X (Le^x), sialyl-Lewis X (sLe^x), Lewis A (Le^a), and sialyl-Lewis A (sLe^a) glycans. Thus, reconstitution of the neutrophil ligand for P-selectin in COS cells requires at least two elements, an enzyme to supply fucose and a specific protein backbone (PSGL-1). PSGL-1 has typical features of an integral membrane mucin, but appears to be uniquely suited for interaction with P-selectin because expression of other mucins in COS cells in the presence of the same fucosyltransferase does not support P-selectin binding (Sako et al., 1993).

In addition to fucose and PSGL-1, additional components of the P-selectin ligand may be inapparent because of their constitutive production in COS cells. For example, both sialic acid and sulfate may be necessary for binding. Neuraminidase treatment of target cells significantly attenuates binding to P-selectin (Corral et al., 1990; Moore et al., 1991), whereas suppression of sulfation on HL-60 cells by exposure to sodium selenate has effects of similar potency (Aruffo et al., 1991). These results raise the possibility that both sulfation in some form and the sLe^x determinant are components of the target structure recognized by P-selectin.

Less is known about P-selectin ligands in mice, but a 160 kDa protein has been identified on neutrophils that binds selectively to a murine P-selectin–immunoglobulin chimera (Lenter et al., 1994), suggesting that specific protein scaffolds may also be required for binding. The murine chimera also recognizes some but not all glycoforms of a low molecular weight mucin-like molecule called CD24–heat stable antigen (Sammar et al., 1994). A correlation was found between those isoforms showing P-selectin binding and those reacting with an antibody recognizing a complex glycan bearing sulfate and glucuronic acid, known as HNK-1 (Sammar et al., 1994).

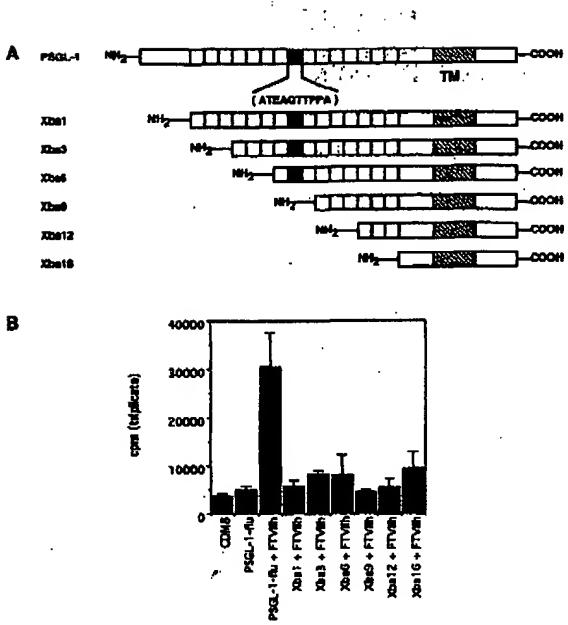
An important topic that remains to be addressed is the

specific sequence features that qualify PSGL-1 as a human P-selectin counterreceptor. In this study, we report an analysis of the elements of PSGL-1 that support P-selectin binding, from which we conclude that a very short element in the apical domain of PSGL-1 contains regulatory elements, including three sites of potential tyrosine sulfation, which are critical for P-selectin ligand function. Treatment of transfected COS cells with chlorate, an inhibitor of sulfation (Baeuerle and Huttner, 1986), partially blocks adhesion to P-selectin but has little effect under the same conditions on binding to E-selectin.

Results

The Amino Terminus of PSGL-1 Is Necessary for P-Selectin Binding

Gross deletions from the amino terminus of the PSGL-1 mucin were prepared by polymerase chain reaction (PCR), and the resulting truncated cDNAs were inserted downstream from a secretory peptide sequence fused to a peptide tag derived from influenza hemagglutinin (HA). Expression plasmids encoding the truncated molecules (Figure 1A) were transfected into COS cells in the presence of a myeloid fucosyltransferase, FTVII, which directs the expression of sLe^x determinants exclusively (Sasaki et al., 1994; Natsuka et al., 1994). Measurement of the binding of radiolabeled transfected cells to plastic wells precoated with P-selectin–immunoglobulin fusion protein showed that deletion of the amino-terminal 100 residues of PSGL-1 was sufficient to abolish binding (Figure 1B). Expression of the deletion variants at the cell surface was confirmed by indirect immunofluorescence using anti-HA monoclonal antibodies, and the presence of sLe^x on the cell surface was confirmed using the monoclonal antibody CSLEX-1 (Figure 1C).



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Construct	Expression (MFI)	
	Flu	SLeX
PSGL-1-flu	5.0	37
Xba1	17	26
Xba3	20	28
Xba6	21	28
Xba9	12	26
Xba12	6.0	30
Xba16	6.0	25

Figure 1. Effect of Amino-Terminal Gross Deletions on PSGL-1 Activity

(A) Schematic structure of the PSGL-1 deletions. Systematic deletion of the ectodomain of PSGL-1 was undertaken by a PCR strategy. A representative 10-residue repeat is shown stippled, and the transmembrane domain is hatched.

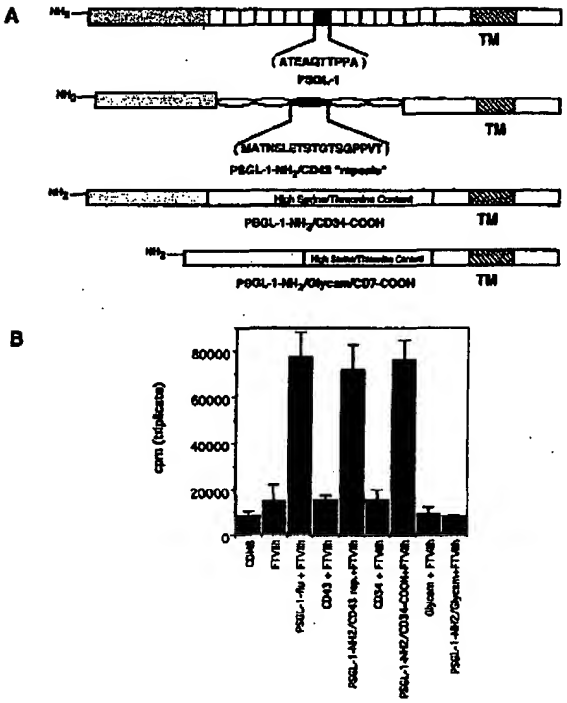
(B) P-selectin-binding activity of transfected COS cells expressing the deletions shown in (A). ^{51}Cr-labeled cells were allowed to adhere to soluble P-selectin adsorbed to microtiter wells and washed, and the bound cells were lysed and counted. Deletion constructs were transfected either in the absence (column 2) or presence (remaining seven columns) of the human FTVII fucosyltransferase (FTVIIh). Error bars denote one standard deviation.

(C) Mean fluorescence intensity (MFI) of COS cells cotransfected with human FTVIIh and the deletion constructs shown in (A) and subjected to indirect immunofluorescence with antibody against the amino-terminal flu peptide or sLe^x.

The Amino Terminus of PSGL-1 Is Sufficient for Binding in the Context of Some but Not All Mucins

To explore whether PSGL-1 sequences other than those in the apical domain of PSGL-1 were required for binding, the transmembrane and cytoplasmic regions of PSGL-1 were replaced with those of the CD43 antigen (Pallant et al., 1989; Shelley et al., 1989). The resulting molecule, which bore no cysteine residues, showed no compromise of binding activity (data not shown). Thus, under these conditions, neither PSGL-1 disulfide bond formation nor a specific membrane-anchoring segment are required for P-selectin binding.

The predicted first 100 amino acids of PSGL-1 were then genetically grafted onto the amino termini or mucin-like repeat elements of a small number of unrelated mucins to determine whether the PSGL-1 apical domain is sufficient for P-selectin counterreceptor activity (Figure 2A). Some but not all mucins proved capable of supporting P-selectin binding in this setting. CD34 and CD43, two relatively large mucins found predominantly on human hematopoietic cells, were both capable of supporting binding, whereas an artificially anchored variant of glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), a mucin expressed on high endothelial venules that has L-selectin ligand activity (Lasky et al., 1992), was inactive in this assay (Figure 2B). The GlyCAM-1 mucin domain in these experiments was tethered to the cell surface via the extracellular stalk, transmembrane, and cytoplasmic anchoring segments of CD7 (Aruffo and Seed, 1987). Cell surface expression of the different mucins and mucin chimeras was confirmed by indirect immunofluorescence using antibodies against flu tag, sLe^x, or the respective mucins (Figure 2C; data not shown).



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Construct	Expression (MFI)	
	Flu	SLeX
FTVIIh	—	52
PSGL-1-flu	9.8	43
CD43	—	60
PSGL-1-NH2/CD43 rep.	12	67
CD34	—	50
PSGL-1-NH2/CD34-COOH	8.0	33
Glycam-flu	12	32
PSGL-1-NH2/Glycam-COOH	8.0	28

Figure 2. P-Selectin-Binding Activity of Chimeric Mucins Bearing the PSGL-1 Apical Domain

(A) Diagram of the constructs. The PSGL-1 N-terminus is stippled, the transmembrane domains are shown hatched, and a representative repeat is shown densely stippled. The PSGL-1 N-terminus was fused to the N-terminus of the predicted mature CD34 and GlyCAM-1 and to the N-terminus of the repeat region of CD43.

(B) P-selectin-binding activity of transfected COS cells expressing either intact mucins, or the amino-terminally derivatized constructs shown in (A). Both native and derivatized GlyCAM-1 are tethered to the membrane through a CD7 stalk and transmembrane domain. FTVIIh, human FTVII fucosyltransferase. CDM8, vector control. Error bars denote one standard deviation.

(C) Mean fluorescence intensity measurement of expression of flu tag or sLe^x by COS cells transfected with the constructs analyzed in (B). CD34 and CD43 constructs were positive for expression by indirect immunofluorescence using the cognate anti-CD antibodies (data not shown).

Mucins That Support Apical Domain-Directed Binding Are Large and Sulfated

The apparent molecular masses determined by denaturing polyacrylamide gel electrophoresis of CD43 and CD34 expressed in COS cells have been reported to be 100–130 kDa (Shelley et al., 1989) and 100 kDa (Simmons et al., 1992), respectively, whereas the PSGL-1 monomer exhibits an effective molecular mass of 110 kDa (Sako et al., 1993). GlyCAM-1, in its native (untethered) state, comigrates with 50 kDa proteins (Lasky et al., 1992). Although it has not been established at present whether a precise estimate of the molecular mass of a mucin-like protein can be achieved by measurement of relative mobility in polyacrylamide gels, nor the appropriate migration–mass relationship, the larger mucins appear to be able to support P-selectin binding when the apical domain of PSGL-1 is appended to the amino terminus.

To explore the possibility that sulfation might be one determinant of the ability of other mucins to support apical domain–directed binding, we evaluated the potential of the different mucins above to undergo sulfation in COS cells. PSGL-1, CD34, CD43, and GlyCAM-1 soluble mucin chimeras readily incorporated sodium [^{35}S]sulfate when expressed in COS cells (Figure 3).

Sequential deletion of the internal repeat elements of PSGL-1 allows one to shorten the molecule in a regular and systematic way with relatively little danger of compromising hypothetical global tertiary associations. As repeat elements were deleted, the binding activity of PSGL-1 declined, consistent with the idea that distance from the plasma membrane might be an important determinant of P-selectin-binding activity (data not shown). Alternatively, other factors, such as carbohydrate density, may contribute to P-selectin binding efficacy.

Inhibition of Sulfation Blocks PSGL-1 Binding to P-Selectin

In previous studies, the sulfation inhibitor sodium selenate was found to compromise binding of HL-60 cells to soluble P-selectin significantly, while having little or no effect on binding of the cells to soluble E-selectin (Aruffo et al., 1991). Although sodium selenate proved too toxic for use in transient expression studies, sodium chlorate, another

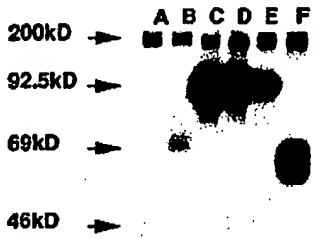


Figure 3. Mucin Sulfation

Mucin–immunoglobulin fusion proteins were labeled with [^{35}S]sulfate, purified on anti-human IgG agarose, and electrophoresed on an 8% denaturing polyacrylamide gel under reducing conditions. Shown is an autoradiogram of the immunoprecipitates of culture supernatants of cells transfected with the following: lane A, CDM8 (vector); lane B, immunoglobulin expression vector (no mucin insert); lane C, PSGL-1–immunoglobulin; lane D, CD43–immunoglobulin; lane E, CD34–immunoglobulin; lane F, GlyCAM-1–immunoglobulin.

relatively selective inhibitor of sulfation (Baeuerle and Huttner, 1986), was effective. COS cells were cotransfected with PSGL-1 and FTVII, or transfected with PSGL-1 and FTVII separately, and incubated for the time period during which maximum synthesis of PSGL-1 was expected in a modified DMEM medium lacking sulfate and containing 10 mM sodium chlorate. Under these conditions, we observed a significant decrease in the ability of cotransfected cells to bind to immobilized P-selectin, whereas the same cells showed little or no decrement in binding to immobilized E-selectin (Figures 4A and 4B). The cell surface expression of either the sLe^x antigen or the PSGL-1 amino-terminal tag sequence was not compromised by this treatment (Figure 4C). Rather, an increase in the mean fluorescence intensity of the transfected cells, for both anti-sLe^x and anti-flu tag, was observed following chlorate treatment. At present, the explanation for this is uncertain. A soluble PSGL-1 immunoglobulin chimera synthesized under comparable conditions showed essentially complete inhibition of [^{35}S]sulfate incorporation (Figure 4D) under conditions in which protein synthesis, as measured by [^{35}S]cysteine and methionine incorporation, was not inhibited (Figure 4E).

Fine Structure Deletion Analysis of the Apical Domain of PSGL-1

To localize elements of the apical domain that contribute to P-selectin counterreceptor activity, we prepared a series of finer deletions across the apical domain, again placing each amino-terminal deletion downstream from the CD5 leader/flu tag element to monitor cell surface expression (Figure 5A). Removal of 20 amino acids from the predicted mature N-terminus had no effect on P-selectin-binding activity, whereas removal of 40 amino acids abrogated binding (Figure 5B). As was the case for the gross deletions, fine structure deletions showed little variability in expression of the epitope tag as assessed by indirect immunofluorescence (Figure 5C). Further deletion gave no change in activity. Thus, sequences between amino acid residues 20–40 (residues 38–57 of the predicted precursor) appear to be required for P-selectin binding.

Sufficiency of a 20 Amino Acid Peptide Fragment for P-Selectin Counterreceptor Activity

To test whether residues 38–57 are sufficient for PSGL-1 apical domain–directed activity, we appended this seg-

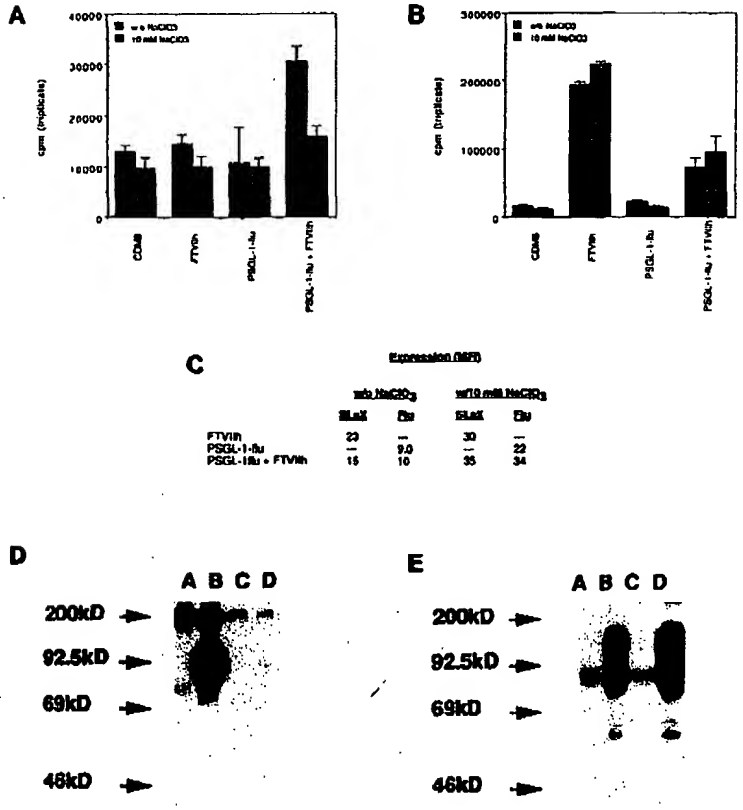


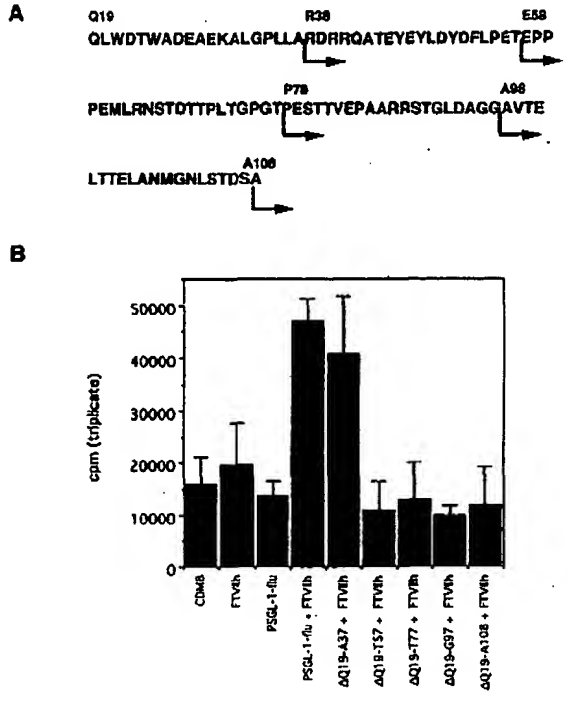
Figure 4. Chlorate Inhibits P- but Not E-Selectin-Mediated Adhesion under Conditions That Do Not Inhibit Protein Synthesis in General

(A) Binding of cells to P-selectin, conducted as described for Figure 1.

(B) Binding of cells to E-selectin under the same conditions.

(C) Chlorate does not inhibit surface expression of PSGL-1 or sLe^x. Shown are the mean fluorescence intensities of COS cells transfected with PSGL-1 with or without FTVII and subjected to indirect immunofluorescence with antibody against the amino-terminal influenza HA peptide or sLe^x.

(D and E) Chlorate inhibits incorporation of [^{35}S]sulfate into soluble mucin chimeras, but does not inhibit protein synthesis in general. Shown are autoradiograms of PSGL-1–immunoglobulin fusion proteins labeled with [^{35}S]sulfate (D) or [^{35}S]cysteine and methionine (E) in the presence or absence of 10 mM $NaClO_3$ and electrophoresed on an 8% denaturing polyacrylamide gel under reducing conditions. Lane A, supernatant of CDM8 transfected cells in the absence of chlorate; lane B, supernatant of cells expressing PSGL-1–immunoglobulin in the absence of chlorate; lane C, supernatant of CDM8 in the presence of chlorate; lane D, supernatant of cells expressing PSGL-1–immunoglobulin in the presence of chlorate.



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Construct	Expression (MFI)
	Flu
PSGL-1-flu	8.5
ΔQ19-A37	12
ΔQ19-T57	14
ΔQ19-T77	15
ΔQ19-G97	15
ΔQ19-A108	15

Figure 5. Sequence and P-Selectin-Binding Activity of Deletion Variants of PSGL-1 Lacking Smaller Elements of the Amino-Terminal Apical Domain

(A) Sequence of PSGL-1 and deletion endpoints. Numbering corresponds to residues in the PSGL-1 precursor (Sako et al., 1993).
(B) P-selectin-binding activity of transfected COS cells expressing the deletion variants with endpoints shown in (A).
(C) Mean fluorescence intensity of COS cells transfected with the deletion constructs shown in (A) and subjected to indirect immunofluorescence with antibody against the amino-terminal influenza HA peptide.

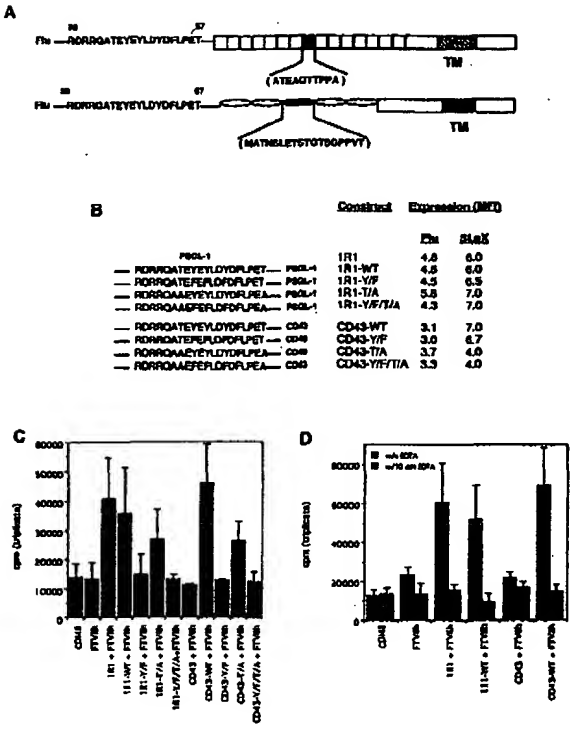

Figure 6. Effect of Appending Wild-Type and Mutant Variants of PSGL-1 Residues 38–57 to Deleted PSGL-1 or CD43

(A) Diagram of the wild-type constructs.
(B) Sequences and nomenclature of the wild-type and mutant variants of PSGL-1 residues 38–57, with the mean fluorescence intensities of the cognate transfectants.
(C) P-selectin-binding activity of transfected COS cells expressing the chimeras shown in (A) and (B). PSGL-1 form 1R1 is full-length PSGL-1 bearing an EcoRI site at the junction of the N-terminal domain and the repeat elements.
(D) Binding of chimeric mucins bearing the amino-terminal 20 residue peptide is sensitive to EDTA.

ment to the amino termini of apical domain–deleted PSGL-1 and CD43 (Figure 6A). In both cases, the addition of this short peptide element conferred P-selectin-binding activity upon the mucin core that was equivalent to that of native PSGL-1 (Figure 6C).

Specific Residues Required within the Amino-Terminal Peptide

Within the 20 amino acid residues necessary for P-selectin binding are three potential tyrosine sulfation sites and two threonine residues. The tyrosines and threonines were subjected to concerted mutation to phenylalanine or alanine, respectively, turning all three tyrosines to phenylalanines and both threonines to alanines. In addition, a quintuple mutation was prepared in which both conversions were made in the same peptide. Each mutated sequence was placed in the same sequence context downstream of the flu tag and upstream of either truncated PSGL-1 lacking the apical domain, or the CD43 repeat elements and transmembrane domain. Cells expressing the resulting chimeras were tested for their ability to bind to immobilized P-selectin. Conversion of the tyrosines to phenylalanines resulted in a loss of binding activity to P-selectin (Figure 6C), without compromise of the expression of the flu tag or sLe^x epitope (Figure 6B). Replacement of the threonine residues with alanine diminished binding, but did not abolish it entirely (Figure 6C). Binding mediated by the apical 20 residues was, like that of native PSGL-1, dependent on the presence of calcium (Figure 6D). P-selectin binding by chimeric mucins bearing the apical 20 amino acid segment was inhibitable by chlorate, whereas E-selectin binding was unaffected under the same conditions (data not shown).

Residues within the Amino-Terminal 20 Amino Acids Are Sulfated on Tyrosine

To determine whether the amino-terminal segment was capable of being sulfated in vivo, we created fusion proteins consisting of the native or mutant peptide sequences joined to human immunoglobulin G1 (IgG1) (Figure 7A). The resulting fusion proteins were expressed in COS cells (Figure 7B), and their ability to assimilate inorganic sulfate was assessed. Immunoglobulin chimeras bearing the native peptide sequences were capable of incorporating sulfate, whereas those bearing phenylalanine substituted for tyrosine were not (Figure 7C). Replacement of threonine with alanine had no effect on sulfate incorporation (Figure 7C).

Inhibitors of Sulfation Block HL-60 Rolling on P-Selectin–Immunoglobulin Chimeras

To explore whether inhibition of sulfation would compromise a physiologically relevant adhesion, we subjected HL-60 cells to growth in medium containing chlorate and examined the ability of the resulting cells to attach and roll on coverslips coated with P-selectin–immunoglobulin chimeras under conditions of defined fluid shear stress (Lawrence et al., 1990). HL-60 cells were capable of attaching to and rolling upon coverslips precoated with P-selectin–immunoglobulin chimera, whereas no such interaction was observed with coverslips coated with a CD4–immunoglobulin chimera (Figure 8; data not shown). Growth of HL-60 cells in chlorate dramatically reduced the frequency of cell interaction with the substrate (Figure 8).

Discussion

The selectin family of cell surface lectins mediates the initial phase of leukocyte–endothelial adhesion, the rolling of cells along activated endothelium (von Andrian et al., 1991; Lawrence and Springer, 1991). Members of the family share a common facility for the recognition of glycans bearing sLe^x determinants (Foxall et al., 1992). However, there are clear differences in the target recognition patterns of the different selectins. E-selectin mediates the binding of both hematopoietic and nonhematopoietic cells bearing sLe^x in a large number of different glycan and protein scaffold contexts (Lowe et al., 1990; Walz et al., 1990; Phillips et al., 1990), suggesting that many proteins

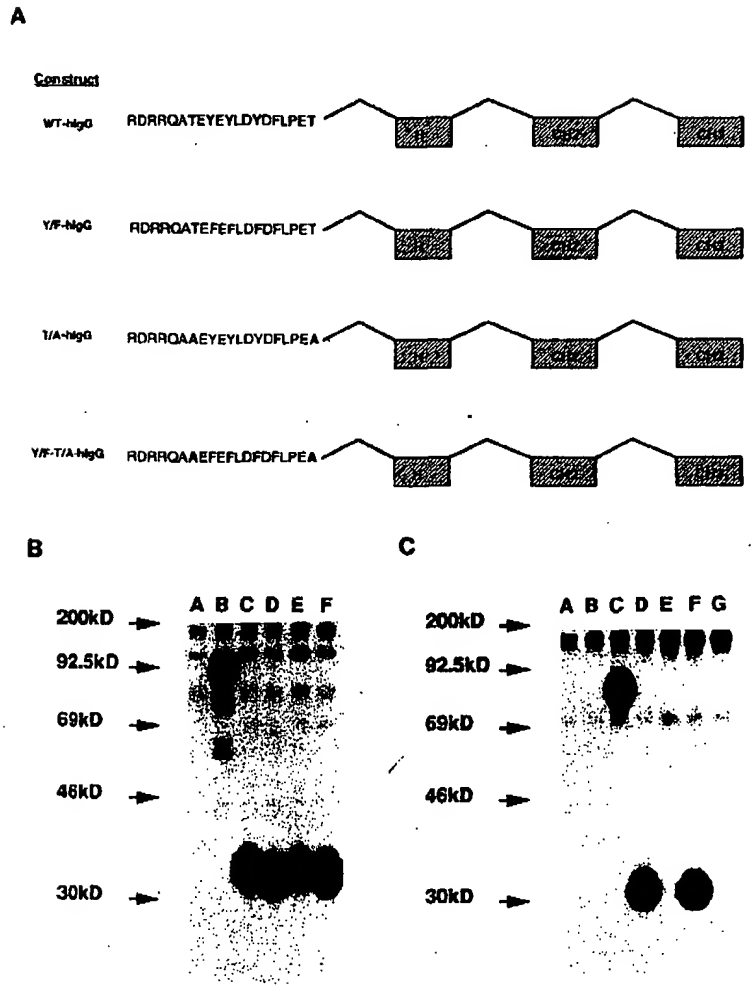

Figure 7. Amino-Terminal Tyrosine Residues Are Sulfated In Vivo

Immunoglobulin fusion proteins consisting of either intact PSGL-1 or 20 residue peptides joined to the hinge, CH2, and CH3 domains of human IgG1 were expressed in COS cells and tested for their ability to assimilate [^{35}S]cysteine and methionine or [^{35}S]sulfate.

(A) Schematic diagram of the peptide fusion constructs.

(B) Incorporation of [^{35}S]cysteine and methionine by the fusion proteins. Lane A, supernatant of cells transfected with CDM8 control. Lane B, supernatant of cells transfected with PSGL-1–immunoglobulin fusion protein. Lane C, supernatant of cells transfected with the WT-hIgG. Lane D, supernatant of cells transfected with Y/F-hIgG. Lane E, supernatant of cells transfected with T/A-hIgG. Lane F, supernatant of cells transfected with Y/F-T/A-hIgG.

(C) Incorporation of [^{35}S]sulfate. The same constructs were tested as in (B), except that a control fusion protein bearing no amino-terminal addition (lane B) was included. Lanes C through G correspond to B through F in (B).

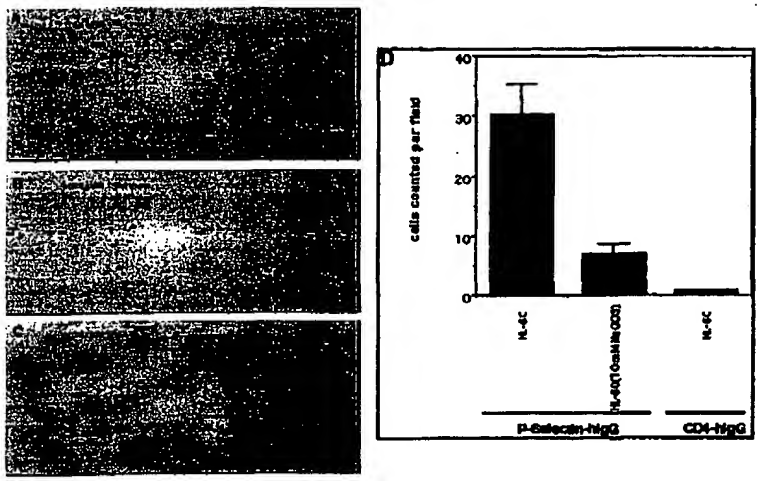

Figure 8. Rolling of HL-60 Cells on P-Selectin–Immunoglobulin Chimeras Is Inhibited by Chlorate

HL-60 cells were infused into a parallel plate flow chamber precoated with either P-selectin–immunoglobulin chimera or a CD4–immunoglobulin chimera control. Cells were subjected to a shear stress of 0.75 dynes/cm².

(A) Video image of HL-60 cells rolling or flowing (streaks) over P-selectin–immunoglobulin chimera.

(B) HL-60 cells pretreated in sulfate-free medium with 10 mM sodium chlorate.

(C) HL-60 cells flowing over coverslip coated with CD4–immunoglobulin chimera.

(D) Tabulation of interacting cells per field from eight frames at 15 s intervals of cells shown in (A)–(C). Error bars represent SEM.

may be capable of presenting sLe^x to E-selectin. These findings do not exclude the possibility that specific proteins on neutrophils are selectively endowed with sLe^x, or possess an enhanced capacity to interact with E-selectin by virtue of their localization to plasma membrane microvilli. Two candidate scaffold proteins that may have superior capacity to present sLe^x to E-selectin are L-selectin on human neutrophils (Picker et al., 1991) and ESL-1, a protein related to fibroblast growth factor, on a murine granulocytic cell line (Steegmaier et al., 1995).

In contrast, P-selectin binding cannot be documented in cells transfected with fucosyltransferases alone, nor in nonhematopoietic cells expressing sLe^x. A major advance in the understanding of P-selectin specificity was the identification of PSGL-1, which has a unique ability (among human neutrophil surface proteins) to support an adhesive interaction with P-selectin (Moore et al., 1992, 1994; Sako et al., 1993). This interaction was shown to require specific glycan modifications that could include sLe^x.

The fucosyltransferase employed for the isolation of PSGL-1 cDNA by expression cloning, FTIII, is a mixed specificity enzyme that synthesizes Le^x, sLe^x, Le^a, and sLe^a (Kukowska-Latallo et al., 1990). In this study, we have demonstrated that coexpression of PSGL-1 in COS cells with the highly specific myeloid lineage enzyme FTVII, which synthesizes sLe^x exclusively (Sasaki et al., 1994; Natsuka et al., 1994), is sufficient for acquisition of P-selectin-binding activity. Thus, it appears that sLe^x is necessary for PSGL-1 cofactor activity and suffices for that activity when it is the only exogenous glycosyltransferase coexpressed with PSGL-1 in COS cells. The necessity for other posttranslational modifications of PSGL-1 that may be required to support P-selectin binding, however, is not ruled out by these studies.

Both L- and P-selectin are known to bind anionic polymers, L-selectin by mannose-6-phosphate polymers, fucoidan, heparin, and related saccharides (Stoolman and Rosen, 1983; Stoolman et al., 1984; Norgard-Sumnicht et al., 1993; Nelson et al., 1993), and P-selectin by heparin, heparan sulfate, dextran sulfate, and fucoidan (Skinner et al., 1989, 1991; Aruffo et al., 1991), as well as related compounds and anionic inositides (Nelson et al., 1993; Cecconi et al., 1994). Both selectins interact strongly with sulfatides (Watson et al., 1990; Imai et al., 1991; Aruffo et al., 1991), 3-sulfated galactosylceramides synthesized by neutrophils and present in a vesicular compartment of nearly all cells undergoing active division either in vivo or in culture (Aruffo et al., 1991; A. Aruffo, W. Kolanus, and B. S., unpublished data). Cells with intracellular sulfatide pools rapidly assimilate inorganic sulfate into glycolipids and excrete them by an as yet uncharacterized mechanism, leading to the rapid accumulation of sulfated lipids in the extracellular space (Aruffo et al., 1991; A. Aruffo, W. Kolanus, and B. S., unpublished data). It has previously been proposed that neutrophil elaboration of sulfated glycolipids represents a mechanism for detachment of granulocytes from P-selectin once they have traversed the endothelium and are prepared to migrate into the organ parenchyma (Aruffo et al., 1991). However, in studies aimed at exploring the role of cell surface sulfatides in P-selectin adhesion, it was also found that sodium selenate, an inhibitor of sulfate assimilation, blocked adhesion of a myeloid cell line (HL-60) to P- but not E-selectin immunoglobulin chimeras (Aruffo et al., 1991). We show here that a similar inhibition of P- but not E-selectin binding can be demonstrated in COS cells expressing PSGL-1 and a sLe^x-specific fucosyltransferase. Moreover, we demonstrate that HL-60 cells subjected to growth in chlorate, an inhibitor of sulfation, have significantly diminished capacity to attach and roll on P-selectin-coated surfaces under conditions of defined flow.

The sequence determinants that distinguish PSGL-1 as a P-selectin counterreceptor appear to be localized within a very short segment at the amino terminus of the molecule, which contains three consensus sites for tyrosine sulfation (Sako et al., 1993; Huttner, 1987) and two possible sites for O-linked glycan addition. This finding is consistent with reports (Ma et al., 1994; Moore et al., 1995) that some monoclonal antibodies that specifically recognize PSGL-1 are capable of blocking binding, a result that sup-

ports the view that the binding site is relatively localized. Elimination of the tyrosines strongly compromises P-selectin binding and concomitantly blocks the incorporation of sulfate into fusion proteins bearing the amino-terminal PSGL-1 domain, suggesting that tyrosine sulfate on the protein backbone, and sLe^x on an O-linked glycan, may be jointly sufficient to constitute P-selectin-binding activity. Some residual activity can be seen among constructs in which the threonines but not tyrosines have been mutated, which may reflect the potential utilization of adjacent threonines of the mucin stalk for glycan presentation. It may also be that both tyrosine and glycan sulfations are required for P-selectin-binding activity. No sulfation of fusion proteins was seen when the tyrosine residues were mutated, but the possibility that glycan and tyrosine sulfations are linked for that sequence context, or that the sulfation of native mucins is independent of tyrosine sulfation, cannot be excluded. Unfortunately, metabolic inhibitors capable of discriminating between tyrosine and glycan sulfations have not been described to our knowledge.

Although studies with N-glycanase have shown that N-linked glycans are dispensable for P-selectin-binding activity (Moore et al., 1992), the possibility that the threonines of the active peptide could be replaced by N-linked glycan addition sites has not been excluded. Nonetheless, the evidence is compelling that both a fucosylated, sialylated glycan and a motif directing the appearance of sulfated tyrosine are important for binding.

Where on P-selectin would these structures interact? The suggestion that two distinct elements may form a site for P-selectin interaction naturally suggests there may be discrete locations on P-selectin that bind to different components of the PSGL-1 ligand. A reasonable hypothesis might be that the tyrosine sulfates interact with some element of the epidermal growth factor (EGF)-like domain, whereas the sLe^x interacts with the lectin domain. Some support for this hypothesis can be found in the report that the L- and P-selectin EGF-like domains are not interchangeable for ligand recognition, suggesting that these domains may directly interact with distinct features of the selectin ligands (Kansas et al., 1994). Concurrent interaction of two or more components of PSGL-1 with P-selectin lectin domain residues may also take place. A more exotic possibility is that the two PSGL-1 components interact with a largely contiguous surface of P-selectin in a stereotyped temporal sequence, for example, in which the glycan forms a rapidly reversible link followed by displacement by, or coalescence with, the tyrosine sulfate site. Finally, sulfation may effect global changes in amino-terminal domain structure that either influence the subsequent disposition of glycans on the apical residues, or affect the conformation of peptide determinants critical for protein–protein interaction.

Experimental Procedures

Production of Soluble P- and E-Selectin Chimeras

P- and E-selectin immunoglobulin chimeras were prepared as previously described (Aruffo et al., 1991; Walz et al., 1990). The PSGL-1 cDNA coding sequence was obtained by PCR from an HL-60 cDNA library and confirmed by DNA sequencing. The coding segment for the mature extracellular, transmembrane, and intracellular domain was inserted in an expression vector based on CDM8 that lacks the polyoma virus origin of replication and contains the leader sequence for the CD5 antigen positioned just upstream of the coding region for a flu peptide (Field et al., 1988) epitope tag (J. Haas and B. S., unpublished data).

Construction of Large Scale PSGL-1 Deletions

Amino-terminal PSGL-1 deletion constructs were prepared by PCR using primers encoding the desired deletion endpoint downstream of an XbaI site in frame two (encodes leucine aspartic acid). The resulting sequences encoded a polypeptide in which the residues listed below immediately followed the aspartic acid (D) of the Xba site: A118, A128, A138, A148, A158, A168, G178, A188, A198, A208, A218, A228, A238, A248, A258, and T268 of the PSGL-1 precursor. The PCR fragments were inserted in the CD5 leader flu tag expression vector used for expression of the intact PSGL-1. The flu tag terminates in an XbaI site in the frame described above. Sequences at the flu tag junction were verified, and expression was confirmed in COS cells by indirect immunofluorescence microscopy and flow cytometry. A series of internal deletions with an EcoRI site at the site of the deletion in frame one (encodes glutamic acid phenylalanine) was also prepared by first creating deletion variants with amino termini (residues immediately following phenylalanine [F] of the EcoRI site) corresponding to A118, A128, A138, A148, A158, A168, G178, A188, A198, A208, A218, A228, A238, A248, and A258 of the peptide sequence of the precursor. To each of these deleted variants was appended a flu-tagged amino-terminal PSGL-1 domain ending with an EcoRI site in the glutamic acid phenylalanine frame immediately downstream of PSGL-1 precursor A117. The resulting constructs contain deletions between A117 and the various endpoints above.

Mucin Domain Interchanges

CD34, CD43, and GlyCAM-1 mucins were prepared for addition of the PSGL-1 amino-terminal domain by appending an EcoRI site to either the mature amino terminus (CD34 or GlyCAM-1), or to the beginning of a region of threonine/proline-rich repeats (CD43). As above, the EcoRI site was in the frame glutamic acid phenylalanine (frame 1). The CD34 sequence began at residue F30 of the precursor, the GlyCAM-1 at precursor L19, and the CD43 at precursor I135. To each of these was appended the flu-tagged PSGL-1 domain terminating in EcoRI as above. The amino terminus and repeat elements of PSGL-1 were appended to the membrane proximal, transmembrane, and intracellular domains of CD43 through an EcoRI site in the glutamic acid phenylalanine frame positioned immediately upstream of the sequences S225 of the CD43 precursor. The complementary fragment from PSGL-1 corresponded to the amino-terminal residues of the precursor up to T267.

Fine Structure Mapping of the Amino-Terminal Domain

A similar strategy was employed for the construction of deletions in the amino-terminal domain, in which PCR generated deletions were formed using primers bearing an Xba site in the leucine aspartic acid frame (frame 2). Immediately downstream of the residues encoding aspartic acid were the PSGL-1 sequences corresponding to precursor R38, E58, P78, and A98. For the definition of the amino-terminal domain, duplex oligonucleotides were synthesized corresponding to the residues between 38 and 57 with the indicated sequence changes to mutate threonine or tyrosine residues to alanine or phenylalanine. All constructs were confirmed by dideoxy sequencing.

Cell Adhesion Assays

Transfected cells were detached from culture dishes with 0.5 mM EDTA in PBS 48–60 hr after transfection and loaded with 100 μl of $^{51}CrO_4$ (1 mCi/ml; DuPont, Boston, MA) in 0.9% NaCl plus 100 μl of medium at 37°C for 1 hr. Loaded cells were washed twice in PBS and resuspended in 0.2% BSA, 0.15 M NaCl, 3 mM $CaCl_2$. Variation in labeling rate was typically minimal between cells prepared in parallel with the same batch of labeled chromate. The labeled cells were incubated in wells of 96-well microculture plates coated with affinity-purified goat anti-human IgG antibody (100 μl of 20 μg/ml anti-human IgG Fc [heavy chain specific] in PBS) for 2 hr in a humid chamber at room

temperature. After washing the plate twice with PBS, additional protein-binding sites were blocked by an overnight incubation with 200 μl of 0.2% BSA in PBS. The plate was washed with PBS four times and incubated with 200 μl of fusion protein supernatants for 2 hr. Following three PBS washes and one wash in 0.2% BSA, 0.15 M NaCl, 3 mM $CaCl_2$, 2 × 10^5 cells/well in 200 μl of 0.2% BSA, 0.15 M NaCl, 3 mM $CaCl_2$ were added and left to bind for 15 min in room temperature while the plate rotated on a rotary platform (80 rpm). The plate was washed three times by carefully filling the wells with 200 μl of 0.15 M NaCl, 3 mM $CaCl_2$ and then inverting the plate on a pile of paper towels. Adherent cells were lysed by the addition of 200 μl of 2% SDS and counted in a γ-ray spectrometer.

Immunofluorescence Analysis

Cells were prepared for cytometry by incubation with the primary monoclonal antibody (typically a 1:200 dilution of ascites, or 5 μg/ml purified antibody) in PBS containing 3% BSA for 30–45 min. The cells were washed twice with PBS and incubated with 2 μg/ml FITC-conjugated affinity-purified antibody to either mouse IgG (12CA5) or mouse IgM (CSLEX-1) for 30–45 min in PBS, 3% BSA, and then washed twice with PBS, resuspended in 1 ml of 1% formaldehyde in PBS prior to analysis. For immunofluorescence microscopy, transfected cells were fixed with 4% freshly depolymerized paraformaldehyde, washed, exposed to BSA at 3% in PBS for 30 min, and then incubated with primary antibody (ascites, 1:250) for 30–45 min, washed twice with PBS, and incubated 30–45 min with FITC-conjugated affinity-purified antibody to mouse IgG (Cappel) at 2 μg/ml in PBS containing 3% BSA. They were then washed twice with PBS and analyzed.

Metabolic Labeling

COS cells transfected with expression plasmids encoding immunoglobulin chimeras were trypsinized the day after transfection and transferred to new plates in complete medium (DMEM with 10% calf serum). Prior to labeling, the medium was removed, washed once with PBS, and replaced with either cysteine and methionine-free medium for labeling with [^{35}S]cysteine and methionine (TransLabel, ICN) or with sulfate-free CRCM-30 medium (Sigma) for labeling with sodium $^{35}SO_4$. Serum was not added, and radionuclide was typically present at a concentration of 200 μCi/ml. After a labeling interval of 12–16 hr, the supernatants were harvested and the fusion proteins collected by adsorption to goat anti-human IgG agarose (Cappel). Adsorbed proteins were subjected to denaturing electrophoresis on 8% polyacrylamide gels under reducing conditions.

Chlorate Inhibition of Adhesion

COS cells were transfected by DEAE dextran and incubated immediately following transfection in DMEM containing 10% calf serum and 10 mM sodium chlorate. After trypsinization a day after transfection, the cells were incubated in fresh dishes in the same medium for 6 hr, whereupon the medium was removed, cells were washed with PBS, and incubated 18 hr further in a custom prepared DMEM medium (Life Technologies) lacking sulfate and containing 2% of the usual levels of cysteine and methionine with 10% dialyzed fetal bovine serum in the presence of 10 mM sodium chlorate (Baeuerle and Huttner, 1986). Cells were then harvested for adhesion and immunofluorescence as described. Control cells were treated similarly, but were incubated in DMEM containing undialyzed serum.

HL-60 Cell Rolling

Video images of HL-60 cells rolling through a parallel plate rectangular flow chamber (FCS2, Bioptechs, Incorporated, Butler, PA) with a temperature controlled stage set at 37°C were acquired with an AIMS Technology (Bronx, NY) CCD camera mounted on a Zeiss ICM 405 inverted microscope equipped with a 2.5× objective. The chamber height was 250 μm. Cells were withdrawn through the chamber at a defined flow rate with the aid of a Harvard Apparatus (South Natick, MA) model I/W 22 syringe pump. Images were analyzed using NIH Image. To inhibit sulfation, HL-60 cells were washed once with PBS and grown for 18 hr in sulfate-free medium containing 2% of the normal levels of cysteine and methionine, 10 mM sodium chlorate, and dialyzed serum as described above. For each experiment, 10^6 cells were suspended in 1 ml of 0.15 M NaCl, 3 mM $CaCl_2$ and drawn through the chamber. Glass coverslips were coated with affinity-purified goat anti-human IgG antibody at a concentration of 10 μg/ml in 50 mM Tris–HCl (pH 9.0) for 2 hr, washed twice with PBS, and blocked overnight with 0.2% BSA in PBS. The treated coverslips were then immersed in supernatants of COS cells transfected with the appropriate immunoglobulin chimera expression plasmids, washed twice with PBS, and assembled in the flow chamber.

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References

Aruffo, A., and Seed, B. (1987). Molecular cloning of two CD7 (T-cell leukemia antigen) cDNAs by a COS cell expression system. EMBO J. *6*, 3313–3316.

Aruffo, A., Kolanus, W., Walz, G., Fredman, P., and Seed, B. (1991). CD62/P-selection recognition of myeloid and tumor cell sulfatides. Cell *67*, 35–44.

Baeuerle, P.A., and Huttner, W.B. (1986). Chlorate: a potent inhibitor of protein sulfation in intact cells. Biochem. Biophys. Res. Commun. *141*, 870–877.

Bajorath, J., Hollenbaugh, D., King, G., Harte, W., Jr., Eustice, D.C., Darveau, R.P., and Aruffo, A. (1994). CD62/P-selectin binding sites for myeloid cells and sulfatides are overlapping. Biochemistry *33*, 1332–1339.

Bonfanti, R., Furie, B.C., Furie, B., and Wagner, D.D. (1989). PADGEM (GMP140) is a component of Weibel–Palade bodies of human endothelial cells. Blood *73*, 1109–1112.

Cacconi, O., Nelson, R.M., Roberts, W.G., Hanasaki, K., Mannori, G., Schultz, C., Ulich, T.R., Aruffo, A., and Bevilacqua, M.P. (1994). Inositol polyanions: noncarbohydrate inhibitors of L- and P-selectin that block inflammation. J. Biol. Chem. *269*, 15060–15066.

Corral, L., Singer, M.S., Macher, B.A., and Rosen, S.D. (1990). Requirement for sialic acid on neutrophils in a GMP-140 (PADGEM) mediated adhesive interaction with activated platelets. Biochem. Biophys. Res. Commun. *172*, 1349–1356.

Erbe, D.V., Watson, S.R., Presta, L.G., Wolitzky, B.A., Foxall, C., Brandley, B.K., and Lasky, L.A. (1993). P- and E-selectin use common sites for carbohydrate ligand recognition and cell adhesion. J. Cell Biol. *120*, 1227–1235.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I.A., Lerner, R.A., and Wigler, M. (1988). Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Foreman, K.E., Vaporciyan, A.A., Bonish, B.K., Jones, M.L., Johnson, K.J., Glovsky, M.M., Eddy, S.M., and Ward, P.A. (1994). C5a-induced expression of P-selectin in endothelial cells. J. Clin. Invest. *94*, 1147–1155.

Foxall, C., Watson, S.R., Dowbenko, D., Fennie, C., Lasky, L.A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B.K. (1992). The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide. J. Cell Biol. *117*, 895–902.

Gamble, J.R., Skinner, M.P., Berndt, M.C., and Vadas, M.A. (1990). Prevention of activated neutrophil adhesion to endothelium by soluble adhesion protein GMP140. Science *249*, 414–417.

Geng, J.G., Bevilacqua, M.P., Moore, K.L., McIntyre, T.M., Prescott, S.M., Kim, J.M., Bliss, G.A., Zimmerman, G.A., and McEver, R.P. (1990). Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature *343*, 757–760.

Hamburger, S.A., and McEver, R.P. (1990). GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood *75*, 550–554.

Hattori, R., Hamilton, K.K., Fugate, R.D., McEver, R.P., and Sims, P.J. (1989a). Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J. Biol. Chem. *264*, 7768–7771.

Hattori, R., Hamilton, K.K., McEver, R.P., and Sims, P.J. (1989b). Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. J. Biol. Chem. *264*, 9053–9060.

Hollenbaugh, D., Bajorath, J., Stenkamp, R., and Aruffo, A. (1993). Interaction of P-selectin (CD62) and its cellular ligand: analysis of critical residues. Biochemistry *32*, 2960–2966.

Hsu-Lin, S., Berman, C.L., Furie, B.C., August, D., and Furie, B. (1984). A platelet membrane protein expressed during platelet activation and secretion: studies using a monoclonal antibody specific for thrombin-activated platelets. J. Biol. Chem. *259*, 9121–9126.

Huttner, W.B. (1987). Protein tyrosine sulfation. Trends. Biochem. Sci. *12*, 361–363.

Imai, Y., Singer, M.S., Fennie, C., Lasky, L.A., and Rosen, S.D. (1991). Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. J. Cell Biol. *113*, 1213–1221.

Kansas, G.S., Saunders, K.B., Ley, K., Zakrzewicz, A., Gibson, R.M., Furie, B.C., Furie, B., and Tedder, T.F. (1994). A role for the epidermal growth factor–like domain of P-selectin in ligand recognition and cell adhesion. J. Cell Biol. *124*, 609–618.

Kubes, P., and Kanwar, S. (1994). Histamine induces leukocyte rolling in post-capillary venules: a P-selectin-mediated event. J. Immunol. *152*, 3570–3577.

Kukowska-Latallo, J.F., Larsen, R.D., Nair, R.P., and Lowe, J.B. (1990). A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group alpha(1,3/1,4)fucosyltransferase. Genes Dev. *4*, 1288–1303.

Larsen, E., Celi, A., Gilbert, G.E., Furie, B.C., Erban, J.K., Bonfanti, R., Wagner, D.D., and Furie, B. (1989). PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell *59*, 305–312.

Lasky, L.A., Singer, M.S., Dowbenko, D., Imai, Y., Henzel, W.J., Grimley, C., Fennie, C., Gillett, N., Watson, S.R., and Rosen, S.D. (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell *69*, 927–938.

Lawrence, M.B., and Springer, T.A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859–873.

Lawrence, M.B., Smith, C.W., Eskin, S.G., and McIntire, L.V. (1990). Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. Blood *75*, 227–237.

Lenter, M., Levinovitz, A., Isenmann, S., and Vestweber, D. (1994). Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. J. Cell Biol. *125*, 471–481.

Lowe, J.B., Stoolman, L.M., Nair, R.P., Larsen, R.D., Berhend, T.L., and Marks, R.M. (1990). ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. Cell *63*, 475–484.

Ma, L., Raycroft, L., Asa, D., Anderson, D.C., and Geng, J.G. (1994). A sialoglycoprotein from human leukocytes functions as a ligand for P-selectin. J. Biol. Chem. *269*, 27739–27748.

Mayadas, T.N., Johnson, R.C., Rayburn, H., Hynes, R.O., and Wagner, D.D. (1993). Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. Cell *74*, 541–554.

McEver, R.P., Beckstead, J.H., Moore, K.L., Marshall-Carlson, L., and Bainton, D.F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel–Palade bodies. J. Clin. Invest. *84*, 92–99.

Moore, K.L., Varki, A., and McEver, R.P. (1991). GMP-140 binds to a glycoprotein receptor on human neutrophils: evidence for a lectin-like interaction. J. Cell Biol. *112*, 491–499.

Moore, K.L., Stults, N.L., Diaz, S., Smith, D.F., Cummings, R.D., Varki, A., and McEver, R.P. (1992). Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J. Cell Biol. *118*, 445–456.

Moore, K.L., Eaton, S.F., Lyons, D.E., Lichenstein, H.S., Cummings, R.D., and McEver, R.P. (1994). The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetyllactosamine. J. Biol. Chem. *269*, 23318–23327.

Moore, K.L., Patel, K.D., Bruehl, R.E., Li, F., Johnson, D.A., Lichenstein, H.S., Cummings, R.D., Bainton, D.F., and McEver, R.P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J. Cell Biol. *128*, 661–671.

Murphy, J.F., and McGregor, J.L. (1994). Two sites on P-selectin (the lectin and epidermal growth factor–like domains) are involved in the adhesion of monocytes to thrombin-activated endothelial cells. Biochem. J. *303*, 619–624.

Natsuka, S., Gersten, K.M., Zenita, K., Kannagi, R., and Lowe, J.B. (1994). Molecular cloning of a cDNA encoding a novel human leukocyte alpha-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis X determinant. J. Biol. Chem. *269*, 16789–16794.

Nelson, R.M., Cecconi, O., Roberts, W.G., Aruffo, A., Linhardt, R.J., and Bevilacqua, M.P. (1993). Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. Blood *82*, 3253–3258.

Norgard, K.E., Moore, K.L., Diaz, S., Stults, N.L., Ushiyama, S., McEver, R.P., Cummings, R.D., and Varki, A. (1993). Characterization of a specific ligand for P-selectin on myeloid cells: a minor glycoprotein with sialylated O-linked oligosaccharides. J. Biol. Chem. *268*, 12764–12774.

Norgard-Sumnicht, K.E., Varki, N.M., and Varki, A. (1993). Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. Science *261*, 480–483.

Paliant, A., Eskenazi, A., Mattei, M.G., Fournier, R.E., Carlsson, S.R., Fukuda, M., and Frelinger, J.G. (1989). Characterization of cDNAs encoding human leukosialin and localization of the leukosialin gene to chromosome 16. Proc. Natl. Acad. Sci. USA *86*, 1328–1332.

Patel, K.D., Zimmerman, G.A., Prescott, S.M., McEver, R.P., and McIntyre, T.M. (1991). Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J. Cell Biol. *112*, 749–759.

Phillips, M.L., Nudelman, E., Gaeta, F.C.A., Perez, M., Singhal, A.K., Hakomori, S.I., and Paulson, J.C. (1990). ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Le^x. Science *250*, 1130–1132.

Picker, L.J., Warnock, R.A., Burns, A.R., Doerschuk, C.M., Berg, E.L., and Butcher, E.C. (1991). The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. Cell *66*, 921–933.

Sako, D., Chang, X.J., Barone, K.M., Vachino, G., White, H.M., Shaw, G., Veldman, G.M., Bean, K.M., Ahern, T.J., Furie, B., Cumming, D.A., and Larsen, G.R. (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. Cell *75*, 1179–1186.

Sammar, M., Aigner, S., Hubbe, M., Schirmacher, V., Schachner, M., Vestweber, D., and Altevogt, P. (1994). Heat-stable antigen (CD24) as ligand for mouse P-selectin. Int. Immunol. *6*, 1027–1036.

Sasaki, K., Kurata, K., Funayama, K., Nagata, M., Watanabe, E., Ohta, S., Hanai, N., and Nishi, T. (1994). Expression cloning of a novel alpha 1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis X carbohydrate determinants in leukocytes. J. Biol. Chem. *269*, 14730–14737.

Shelley, C.S., Remold-O'Donnell, E., Davis, A.E., Bruns, G.A., Rosen, F.S., Carroll, M.C., and Whitehead, A.S. (1989). Molecular characterization of sialophorin (CD43), the lymphocyte surface sialoglycoprotein defective in Wiskott–Aldrich syndrome. Proc. Natl. Acad. Sci. USA *86*, 2819–2823.

Simmons, D.L., Satterthwaite, A.B., Tenen, D.G., and Seed, B. (1992). Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. J. Immunol. *148*, 267–271.

Skinner, M.P., Fournier, D.J., Andrews, R.K., Gorman, J.J., Chesterman, C.N., and Berndt, M.C. (1989). Characterization of human platelet GMP-140 as a heparin-binding protein. Biochem. Biophys. Res. Commun. *164*, 1373–1379.

Skinner, M.P., Lucas, C.M., Burns, G.F., Chesterman, C.N., and Berndt, M.C. (1991). GMP-140 binding to neutrophils is inhibited by sulfated glycans. J. Biol. Chem. *266*, 5371–5374.

Steegmaier, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H.P., Kleuser, B., and Vestweber, D. (1995). The E-selectin ligand ESL-1 is a variant of a receptor for fibroblast growth factor. Nature *373*, 615–620.

Stenberg, P.E., McEver, R.P., Shuman, M.A., Jacques, Y.V., and Bainton, D.F. (1985). A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. J. Cell Biol. *101*, 880–886.

Stoolman, L.M., and Rosen, S.D. (1983). Possible role for cell-surface carbohydrate-binding molecules in lymphocyte recirculation. J. Cell Biol. *96*, 722–729.

Stoolman, L.M., Tenforde, T.S., and Rosen, S.D. (1984). Phosphomannosyl receptors may participate in the adhesive interaction between lymphocytes and high endothelial venules. J. Cell Biol. *99*, 1535–1540.

Thorlacius, H., Raud, J., Rosengren-Beezley, S., Forrest, M.J., Hedqvist, P., and Lindbom, L. (1994). Mast cell activation induces P-selectin-dependent leukocyte rolling and adhesion in postcapillary venules *in vivo*. Biochem. Biophys. Res. Commun. *203*, 1043–1049.

Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M., and Seed, B. (1990). Recognition by ELAM-1 of the sialyl-Le^x determinant on myeloid and tumor cells. Science *250*, 1132–1135.

Watson, S.R., Imai, Y., Fennie, C., Geoffroy, J.S., Rosen, S.D., and Lasky, L.A. (1990). A homing receptor–IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules. J. Cell Biol. *110*, 2221–2229.

von Andrian, U.H., Chambers, J.D., McEvoy, L.M., Bargatze, R.F., Arfors, K.E., and Butcher, E.C. (1991). Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins *in vivo*. Proc. Natl. Acad. Sci. USA *88*, 7538–7542.

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A Sulfated Peptide Segment at the Amino Terminus of PSGL-1 Is Critical for P-Selectin Binding

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Summary

P-selectin glycoprotein ligand 1 (PSGL-1) is a mucin-like glycoprotein expressed on the surface of myeloid cells and serves as the high affinity counterreceptor for P-selectin. The PSGL-1–P-selectin interaction is calcium dependent and requires presentation of sialyl-Lewis^x (sLe^x)-type structures on the O-linked glycans of PSGL-1. We report here the identification of a non-carbohydrate component of the binding determinant that is critical for high affinity binding to P-selectin. Located within the first 19 amino acids, this anionic polypeptide segment contains at least one sulfated tyrosine residue. We propose that this sulfotyrosine-containing segment of PSGL-1, in conjunction with sLe^x presented on O-linked glycans, constitutes the high affinity P-selectin-binding site.

Introduction

The selectins are a family of calcium-dependent adhesion receptors that can mediate the initial "rolling" interaction between leukocytes and vascular endothelium prior to leukocyte extravasation (Lasky, 1992; Varki, 1994). P-selectin, E-selectin, and L-selectin are structurally similar, and each can bind to related fucosylated or sialylated tetrasaccharide structures (or both), such as sialyl-Lewis^x (sLe^x) or sialyl-Lewis^a (Foxall et al., 1992). Additionally, functional binding to P- and L-selectin is maintained after the substitution of sulfate for sialic acid in these oligosaccharides (Yuen et al., 1992; Brandley et al., 1993). Despite this common binding of sLe^x and related structures, it has become clear that the selectins have distinct adhesion patterns and different affinities for sLe^x-bearing glycoprotein ligands (Varki, 1994).

The human myeloid cell line HL-60 has been shown to display sLe^x-conjugated surface molecules capable of binding both P- and E-selectin (Zhou et al., 1991). Protease treatment of HL-60 cells abolished the binding of P-selectin but not E-selectin, suggesting that sLe^x alone is insufficient for P-selectin binding and some additional component is required (Larsen et al., 1992). A unique glycoprotein ligand consisting of a homodimer with two disulfide-linked subunits of apparent molecular mass ~120 kDa was identified from HL-60 cell membrane extracts following affinity purification using immobilized P-selectin (Moore et al., 1992). This ligand appears to share identity with P-selectin glycoprotein ligand 1 (PSGL-1), a glycoprotein cloned and characterized from an HL-60 cell cDNA library by using an expression cloning strategy employing P-selectin as a panning reagent (Sako et al., 1993). Coexpression of the PSGL-1 cDNA with a cDNA encoding α(1,3/1,4)fucosyltransferase (FT3) (Lowe et al., 1990) in COS cells produced functional PSGL-1 that binds to P-selectin in a calcium-dependent manner. Antibodies raised against this recombinant PSGL-1 recognize the major P-selectin-binding protein purified from HL-60 cell membranes (Sako et al., 1993). PSGL-1 also appears to be the critical calcium-dependent ligand for E-selectin in HL-60 cells (Asa et al., 1995).

Glycosidase digestion of myeloid and soluble recombinant forms of PSGL-1 revealed the presence of O-linked and N-linked oligosaccharides (Sako et al., 1993; Moore et al., 1994). These experiments suggested a key role for O-linked, but not N-linked, oligosaccharides in the binding to P-selectin.

At the outset of this study, we sought to identify the unique structural features of PSGL-1 that enable specific recognition by P-selectin. We began by mapping the location of sLe^x-containing O-linked oligosaccharides present on PSGL-1 that are essential for its binding to P-selectin. Inspection of the amino acids encoded by the cDNA indicated that the central portion of the extracellular domain of PSGL-1 contains 15 decameric repeats that are especially rich in prolines and threonines; additional serines and threonines in proximity of prolines extend from the repeats to the amino terminus. Because O-linked oligosaccharide attachment sites are often found at serine and threonine residues proximal to proline residues in glycoproteins (Wilson et al., 1991), we elected to follow a deletion strategy and remove increasing amounts of the putative O-linked oligosaccharide attachment sites. This was done by generating a series of carboxy-terminal truncated mutants of PSGL-1, each of which is fused to a human immunoglobulin G (IgG) Fc domain. The resulting series of soluble dimeric chimeras was used to delineate the minimal domains required for P- and E-selectin binding. We also noted the unusual presence of three potential sites for tyrosine sulfation found near the amino terminus of mature PSGL-1 (Sako et al., 1993). Using site-directed mutagenesis, we have assessed their functional importance in P- and E-selectin binding.

Our results demonstrate that the first 19 amino acids of PSGL-1, containing only a single O-linked oligosaccharide, are capable of binding to P-selectin. We show that this region contains at least one sulfated tyrosine residue that is necessary for high affinity binding to P-selectin. In addition, differential in vitro binding of E- and P-selectin is observed with specific mutated forms of PSGL-1.

*These authors contributed equally to this work.

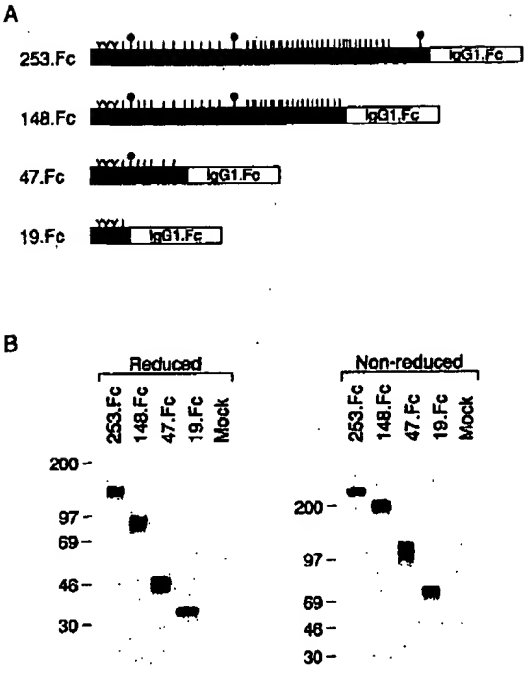

Figure 1. Structure of PSGL-1 Chimeras

(A) Schematic representation of the chimeric PSGL-1–IgG deletion series. The closed bars indicate PSGL-1 segments, and the open bars represent IgG segments. Y, amino-terminal tyrosine. Vertical lines denote the approximate number and location of O-linked oligosaccharides. Vertical lines with closed circles represent locations of potential N-linked glycosylation sites.

(B) Autoradiograph of the series of soluble PSGL-1–IgG chimeras. COS cells were transiently transfected with plasmids encoding sPACE, 3/4FT, and the chimera. Conditioned medium containing the [^{35}S]methionine-labeled chimeras was precipitated using protein A–Sepharose and resolved on SDS–polyacrylamide gels under either reducing or nonreducing conditions as indicated. Mock lanes refer to cells transfected only with sPACE and 3/4FT plasmids. Numbers on the left column correspond to molecular mass markers.

Results

Recombinant PSGL-1–Fc Chimeras Are Processed and Secreted as Soluble Dimers

Previously, we have shown that a soluble form of PSGL-1, sPSGL-1.T7, encoding the extracellular amino-terminal 254 amino acids fused to an epitope tag sequence, is capable of binding both P- and E-selectin in a calcium-dependent fashion (Sako et al., 1993). To define the binding epitope for P-selectin, we fused various lengths of the extracellular domain of PSGL-1 to the heavy chain C_H2–C_H3 region of IgG1 (Capon et al., 1989). This chimeric format facilitated formation of soluble dimers and enabled rapid purification by protein A–Sepharose.

As shown schematically in Figure 1A, 253.Fc is the longest of these chimeric fusions and, like sPSGL-1.T7, contains all potential O- and N-linked glycosylation sites. The shortest chimera, 19.Fc, contains only the first 19 amino acids and no N-linked glycosylation sites. Expression plasmids encoding these chimeras were cotransfected into COS cells with a plasmid encoding an FT3 enzyme (Lowe et al., 1990) and a plasmid encoding a soluble form of the paired basic amino acid converting enzyme (PACE) (Rehemtula and Kaufman, 1992; Wasley et al., 1993). The rationale for including the PACE was predicated on amino-terminal amino acid sequencing results. Purified recombinant PSGL-1.T7 produced from COS cells yields a peptide sequence QATEYE, confirming the utilization of the PACE consensus sequence (R. Ettling, H. White, data not shown). Therefore, the PACE plasmid was included in the cotransfection experiments to ensure complete processing of PSGL-1. Figure 1B demonstrates that all of the truncated forms of PSGL-1 resulting from cotransfections in COS cells were secreted as disulfide-linked soluble homodimers.

Binding of Truncated PSGL-1–Fc Chimeras to P- and E-Selectin

Two different assays were used to monitor the binding of PSGL-1 constructs to P- and E-selectin. The first of these employs soluble chimeric forms of P-selectin or E-selectin covalently coupled to agarose beads and measures the binding of [^{35}S]methionine-labeled PSGL-1 chimeras. Only interactions with sufficient affinity to withstand multiple washing steps yield demonstrable binding. The behavior in this assay of the longest PSGL-1 construct, 253.Fc, is consistent with previous studies of PSGL-1 isolated from human neutrophils (Moore et al., 1992, 1994; Norgard et al., 1993). The protein can be reprecipitated by a polyclonal PSGL-1 antibody after purification on protein A–Sepharose and treatment with sialidase, yet no longer can be affinity captured by P-selectin (Figure 2A). Experiments in which a fucosyltransferase enzyme is omitted from COS cell cotransfections demonstrate the dependence of the protein on fucosylation for activity as well (Figure 2A). In all experiments, recombinant PSGL-1 mutants bound to the P-selectin resin are eluted with EDTA and EGTA, demonstrating cation (presumably calcium) dependence of the interaction. The interaction therefore appears to have a dependence on sLe^x or a related structure. A titration of the amount of 253.Fc-conditioned medium added into this assay indicates a linear dose response over as much as a 40-fold range, as monitored by autoradiography (Figure 2A) and phosphorimager quantitation.

Figure 2B shows that each of the truncated forms of PSGL-1 binds to P-selectin with sufficient affinity to be detected in this assay. Surprisingly, even the 19.Fc chimera, comprising only the first 19 amino acids of PSGL-1, exhibits binding to P-selectin. Phosphorimager analysis indicates that binding of the 19.Fc form to P-selectin occurs to approximately 5% the extent of binding of the 253.Fc form. For the E-selectin chimera, only the 253.Fc and 148.Fc chimeras are observed to bind with comparable affinity. Trace amounts of the 47.Fc chimera are detected to bind E-selectin, and the 19.Fc construct demonstrates no detectable binding. Essentially identical results also are obtained from constructs cotransfected with the recently cloned myeloid α(1,3)fucosyltransferase, FT7 (Natsuka et al., 1994; Sasaki et al., 1994) (data not shown).

In the second assay, CHO cells expressing full-length

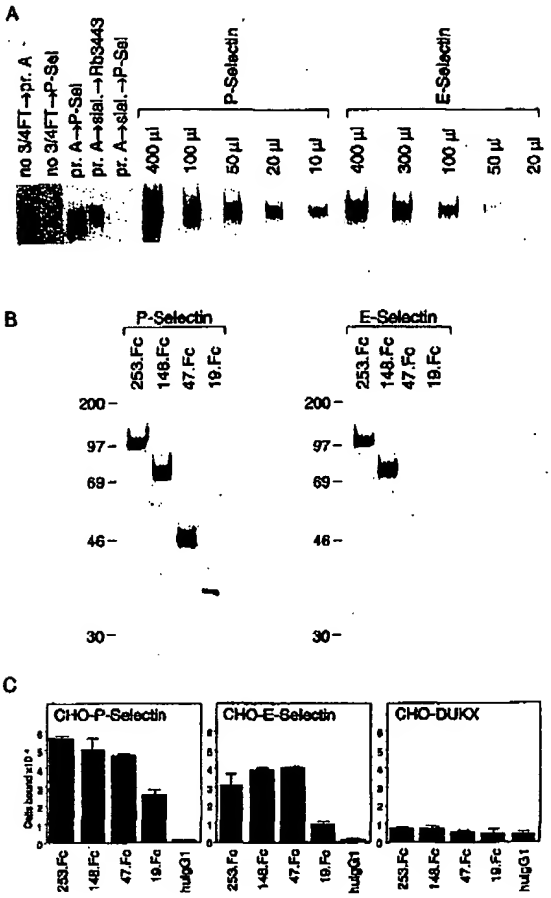

Figure 2. Selectin Binding Studies of PSGL-1 Chimeras

(A) Autoradiograph of 253.Fc protein affinity capture experiments. The left five lanes show the effect on P-selectin affinity capture of cotransfection with and without the vector encoding a fucosyltransferase enzyme (3/4FT), as well as the effect of treatment with sialidase. The [^{35}S]methionine-labeled PSGL-1–IgG chimera was purified from conditioned medium with protein A–Sepharose (pr. A) prior to treatment with sialidase (sial.). The remaining lanes show the effects on P- and E-selectin affinity capture of incubating various amounts of conditioned medium with the capture resin. COS cell–conditioned media containing the protein were precipitated using selectin–IgG chimeras coupled to agarose beads, eluted with EDTA and EGTA, and run on SDS–polyacrylamide gel under reducing conditions.

(B) Autoradiograph of PSGL-1–IgG chimera series affinity captured by P- and E-selectin and eluted with EDTA and EGTA as above. Numbers on the left column correspond to molecular mass markers.

(C) Cell binding assay. Microtiter plate wells were coated with purified PSGL-1–IgG chimeras or human IgG antibody at 2 µg/ml and incubated with ^{3}H-thymidine-labeled CHO cells expressing membrane-bound P-selectin, E-selectin, or negative control (DUKX) cells.

membrane-bound P- and E-selectin (Larsen et al., 1991) were assayed for their binding to microtiter plate wells coated with the PSGL-1 chimeras. This assay allows multivalent interactions; binding events with intrinsic affinities as low as the millimolar range therefore can yield demonstrable binding. Figure 2C shows the results of this assay. As expected, PSGL-1 chimeras that exhibit activity in the higher affinity capture assay also support binding in this assay. In addition, the two chimeras that show little or no higher affinity binding to E-selectin do support binding in this multivalent system. Indeed, in this system the 47.Fc construct is able to support binding at a level indistinguishable from 253.Fc or 148.Fc. Only background levels of binding to any of the PSGL-1 chimeras are observed for control CHO–DUKX cells (Figure 2C). Again, similar results are observed when cotransfections are carried out with FT7 instead of FT3. Taken together, the results from these two binding assays demonstrate that even the first 19 amino-terminal residues of PSGL-1 contain a determinant important for high affinity P-selectin binding and that differential binding of E- and P-selectin could be observed among the various PSGL-1 constructs.

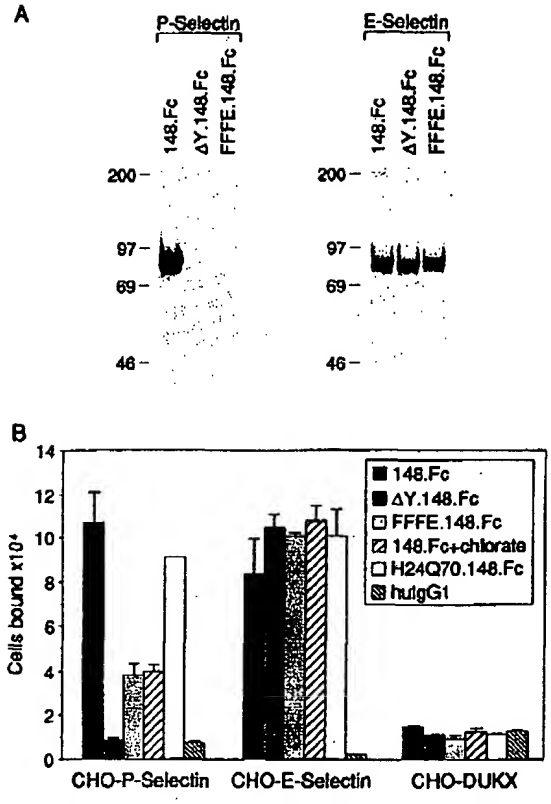

Figure 3. Selectin Binding Studies of PSGL-1 148.Fc Chimera Mutants

(A) Autoradiograph of PSGL-1–IgG chimera mutants affinity captured by P-selectin or E-selectin by method described in Figure 2A.

(B) Cell binding assay of PSGL-1–IgG chimera mutants by method described in Figure 2B.

Sulfation, but Not N-Glycosylation, Is Required for PSGL-1 Binding to P-Selectin

The 148.Fc form of PSGL-1 was chosen for the next series of studies because it is the shortest chimera that supports the higher affinity binding of both P- and E-selectin. To confirm directly that N-linked oligosaccharides on PSGL-1 are not required for selectin binding, we mutated the two remaining putative N-linked glycosylation sites within 148.Fc, producing the mutant H24Q70.148.Fc. The cell-based binding assay was used to show that this mutant remains fully capable of binding to both P- and E-selectin (Figure 3B). This result confirms previous studies em-

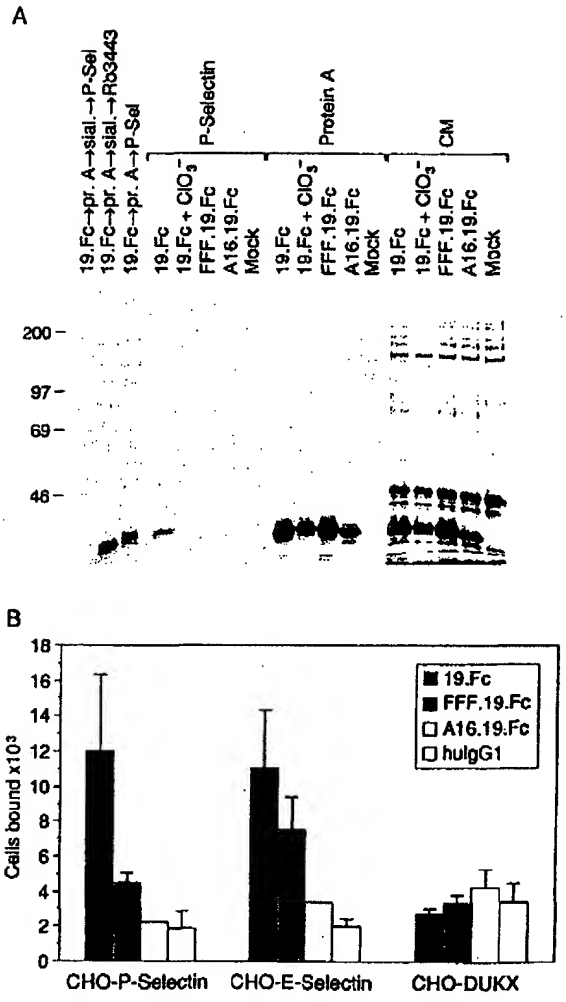

Figure 4. Selectin Binding Studies of PSGL-1 19.Fc Chimera Mutants

(A) Autoradiograph of 19.Fc chimera mutants affinity captured by P-selectin, protein A–Sepharose, or conditioned medium (CM), as indicated above the lanes. Chlorate-treated cells are indicated (ClO_3^-).

(B) Cell binding assay of 19.Fc chimeras, by method described in Figure 2B.

ploying peptide N-glycosidase on both recombinant and natural PSGL-1 (Sako et al., 1993; Moore et al., 1994). In addition, the present results suggest that the N-linked oligosaccharides on PSGL-1 also are not required for E-selectin binding.

Next, we examined whether sulfation of PSGL-1 is important for its binding to P-selectin. A metabolic inhibitor of ATP sulfurylase activity, sodium chlorate (Lipmann, 1958), was used to prevent sulfation of both natural PSGL-1 on myeloid cells and recombinant PSGL-1 in COS cells. Papain-treated HL-60 cells were incubated in the presence or absence of 50 mM chlorate for 48 hr. Analysis by fluorescence-activated cell sorting, employing fluorescently labeled selectin chimeras, demonstrated a significantly decreased level of P-selectin reactivity with PSGL-1 on the surface of chlorate-treated cells. Surface expression of PSGL-1 on these cells was confirmed with a fluorescently labeled monoclonal antibody to PSGL-1 (data not shown).

Sodium chlorate treatment of COS cells producing the 148.Fc protein also yielded PSGL-1 with markedly reduced P-selectin binding (Figure 3B), indicating the importance of sulfation for PSGL-1 function. In contrast, E-selectin binding was completely unaffected by sodium chlorate treatment of myeloid cells or COS cells expressing 148.Fc (Figure 3B).

The Critical Sites of PSGL-1 Sulfation Reside on the Amino-Terminal Tyrosines

Because sodium chlorate inhibits both carbohydrate and tyrosine sulfation, the sites of critical sulfation could be on O-linked oligosaccharides, one or more of these tyrosine residues, or both. Since the first 14 residues of PACE-processed PSGL-1 contain a region with several potential sites of tyrosine sulfation (Huttner and Baeuerle, 1988), the

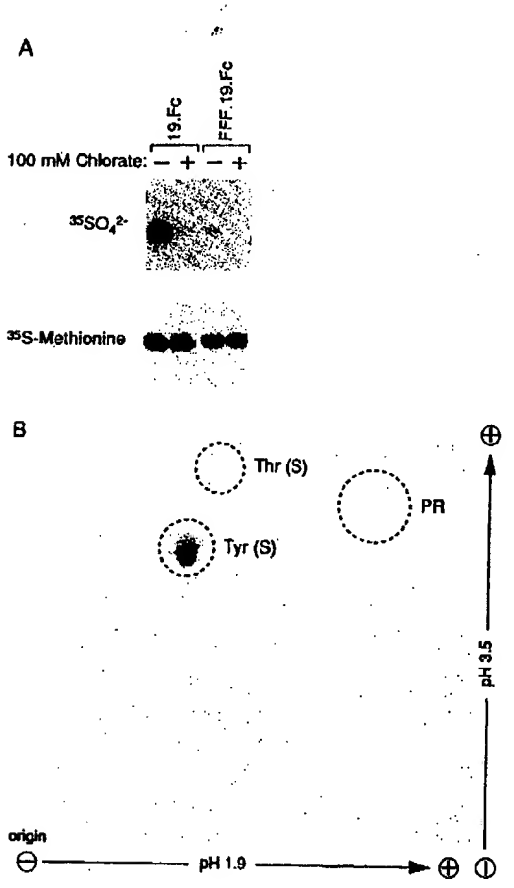

Figure 5. Analysis of Sulfate in PSGL-1 19.Fc Chimeras

(A) Autoradiograph showing sulfate incorporation into 19.Fc chimera proteins. Conditioned medium from cells fed with either [^{35}S]sulfate or [^{35}S]methionine, in the presence and absence of sodium chlorate, was affinity precipitated using protein A–Sepharose, and the proteins were resolved by reducing SDS–polyacrylamide gel electrophoresis.

(B) Thin-layer electrophoresis to detect tyrosine sulfate. A [^{35}S]sulfate-labeled and protein A–purified 19.Fc chimera sample was subjected to alkaline hydrolysis with barium hydroxide and analyzed by two-dimensional gel electrophoresis at pH 1.9 and 3.5. The autoradiograph is shown, and the locations of unlabeled tyrosine sulfate (Tyr(S)), threonine sulfate (Thr(S)), and phenol red (PR) markers are denoted by dashed circles.

148.Fc chimera was used to create two types of specific mutations in the region of putative tyrosine sulfation. An internal deletion mutant, ΔY148.Fc, was created in which the seven amino acids from positions 5–11 of the mature extracellular domain were removed. The deleted region contains three putative tyrosine sulfation sites. This mutant fails to bind P-selectin in either binding assay, yet E-selectin binding is fully maintained (Figures 3A and 3B). The second mutant, FFFE.148.Fc, replaces all three tyrosines, at positions 5, 7, and 10, with phenylalanine and the aspartic acid at position 11 with a glutamic acid. This mutant does show some binding to P-selectin, but only in the multivalent assay. The level of binding is similar to that found after chlorate treatment of the 148.Fc form (Figure 3B). Binding to E-selectin in either assay was completely unaffected by the quadruple mutation. It should be noted that in the lower affinity assay an additional contribution of the acidic residues flanking the tyrosines is indicated (Figure 3B). The data therefore suggest that both the amino-terminal tyrosines and the flanking acidic residues are important specifically for PSGL-1 binding to P-selectin but not E-selectin. To determine whether the amino-terminal tyrosines of PSGL-1 are sulfated, we utilized the 19.Fc chimera in further experiments. Expression of this protein in the presence of chlorate completely abolished binding to P-selectin in the higher affinity assay (Figure 4A). Control precipitations with protein A–Sepharose show that the protein still was synthesized. A triple mutant, termed FFF.19.Fc, also was created to convert each of the three tyrosines at positions 5, 7, and 10 into phenylalanines. Figure 4 shows that, as was seen for FFFE.148.Fc mutant, this mutant fails to bind P-selectin in the higher affinity binding assay. Again, however, a low level of binding is observed in the multivalent cell binding assay. Interestingly, a significant level of E-selectin binding with FFF.19.Fc is still detected in the cell binding assay (Figure 4B).

The residual binding of the FFF.19.Fc mutant to the selectins is likely to be due to an sLe^x-modified O-linked oligosaccharide at Thr-3 or Thr-16. Omitting the plasmid encoding a fucosyltransferase enzyme during COS cell transfection yields a 19.Fc molecule unable to support P- or E-selectin binding (data not shown). Treatment with sialidase also abrogates P-selectin-binding activity (Figure 4A). We created a mutant (A16.19.Fc) in which an alanine residue replaces the threonine at position 16. This mutant exhibits a small shift in gel mobility as compared with 19.Fc, consistent with a loss of oligosaccharide. Moreover, this mutant is unable to support binding to either P- or E-selectin (Figure 4). These data strongly suggest that an O-linked oligosaccharide bearing sLe^x and located at Thr-16 also is essential for 19.Fc binding to P- and E-selectin.

Figure 5 provides evidence that sulfation occurs exclusively on the tyrosines. Examination of [^{35}S]sulfate incorporation by autoradiography shows that far more labeled sulfate is incorporated into 19.Fc than into FFF.19.Fc (Figure 5A). The small amount of label incorporated in the FFF.19.Fc is comparable with levels observed for other unrelated polypeptides fused into the IgG constant region (data not shown) and therefore is likely to represent incorporation into the IgG region rather than into the oligosaccharide at Thr-16. Treatment with sodium chlorate at concentrations that inhibit binding to P-selectin also inhibited the incorporation of sulfate into the 19.Fc chimera. The chlorate effect is specific, as parallel labelings using [^{35}S]methionine indicate that synthesis and secretion of the chimeras in treated or untreated cells are nearly equivalent (Figure 5A).

To confirm directly the presence of sulfated tyrosine, [^{35}S]sulfate-labeled 19.Fc protein was excised from an SDS gel, subjected to alkaline hydrolysis, and then analyzed by two-dimensional thin-layer electrophoresis. Figure 5B shows a single spot from the labeled, hydrolyzed material that comigrates with a sulfated tyrosine standard. At least one sulfated tyrosine therefore is present in PSGL-1.

Discussion

While several glycoproteins have been reported to bind in a specific fashion to L-selectin (Lasky et al., 1992; Baumhueter et al., 1993; Berg et al., 1993), PSGL-1 is the only high affinity P-selectin ligand characterized to date and appears to account for all of the high affinity P-selectin-binding sites on human neutrophils (Moore et al., 1995). Monomeric P-selectin has been reported to bind to ~25,000 sites per neutrophil with an apparent K_d of ~70 nM (Ushiyama et al., 1993). The focus of our work has been to determine what structural features of PSGL-1 differentiate its activity from other sialomucins or glycoproteins.

The results of the mutagenesis studies presented here indicate that an anionic amino-terminal peptide segment of PSGL-1 provides an essential component for the specific high affinity interaction of PSGL-1 with P-selectin. This region is comprised of several acidic amino acid residues and three tyrosines. Furthermore, sulfation of tyrosine residues within this anionic peptide segment is critical for the high affinity binding of P-selectin. This modified region alone is insufficient for high affinity binding, but in concert with an O-linked oligosaccharide modified by sLe^x or a related structure it provides the high affinity recognition determinant for P-selectin. It is noteworthy that sulfation of O-linked glycans rather than tyrosine residues has been shown to be critical for the binding of glycosylation-dependent cell adhesion molecule 1 to L-selectin (Imai et al., 1993; Hemmerich et al., 1994, 1995; Hemmerich and Rosen, 1994).

The profound effect of tyrosine to phenylalanine substitutions on activity may be due solely to removal of the substrate for sulfation or a combination of substrate removal and functional significance of the tyrosine side chain itself. While it is also possible that these mutations are acting indirectly, by altering the sLe^x modification of carbohydrate on the ligand, this is unlikely, since the carbohydrate components of the ΔY148.Fc, FFFE.148.Fc, and FFF.19.Fc mutants are unchanged in their ability to

bind to E-selectin. E-selectin binding indicates the presence of functional sLe^x (or related structures) on the O-linked oligosaccharides of these chimeric proteins, as only glycoproteins modified with sLe^x (or related structures) exhibit binding to E-selectin in vitro.

Our studies are based on PSGL-1 produced in COS cells in the presence of FT3. The use of the myeloid fucosyltransferase, FT7, yields similar results. A potential concern still arises that this COS cell–produced PSGL-1 differs substantially from the native PSGL-1 found on the surface of human neutrophils. While we cannot rule out some structural differences in the oligosaccharides, the validity of the COS cell–produced material for the type of analysis presented here is strongly reinforced by the unique recognition of the recombinant PSGL-1 by P-selectin. Other neutrophil sialoglycoproteins produced in the analogous manner fail to show P-selectin binding (Sako et al., 1993). Polyclonal antisera raised against COS-produced PSGL-1 can block the adherence of HL-60 cells to P-selectin in vitro (Vachino et al., 1995). Chlorate treatment of HL-60 cells reduces the level of binding to P-selectin, but not to E-selectin. Finally, labeled sulfate is incorporated in PSGL-1 isolated from human myeloid U937 cells (R. T. C., J. Flannery, and D. S., unpublished data). Given these similar characteristics between native and recombinant PSGL-1, we believe that the recombinant PSGL-1 is a valid test system and reflects the functional importance of sulfated tyrosines in native PSGL-1.

Previous attempts to assess the molecular determinants of P-selectin ligand recognition have yielded data consistent with unique presentation of oligosaccharides being the sole critical component. One hypothesis, proposed by McEver and colleagues (Norgard et al., 1993) and expanded upon in a recent review (Varki, 1994) is that high affinity selectin–ligand interactions result from a multivalent presentation of sLe^x-modified oligosaccharides. In the specific case of PSGL-1, a "clustered saccharide patch" presented by unique high density O-linked oligosaccharides may be responsible for the observed high affinity and specific binding to P-selectin. While the apparent increase in binding observed between 19.Fc and 148.Fc is consistent with the notion that more than one O-linked oligosaccharide is required for the full binding activity of PSGL-1, this observation is also consistent with the idea that the optimal O-linked oligosaccharide is not localized at Thr-16. Since the ΔY148.Fc and FFFE.148.Fc constructs contain multiple O-links yet fail to bind to P-selectin, clustered oligosaccharides alone cannot constitute the high affinity recognition epitope.

The results presented here argue convincingly for a multicomponent recognition motif. Figure 6 shows a schematic model for this interaction in which both a carbohydrate moiety and an anionic sulfated polypeptide near the amino terminus are necessary for high affinity binding to P-selectin. In contrast, E-selectin binding does not require the anionic peptide portion of the PSGL-1-binding determinant.

The sulfated anionic region resembles that of other sulfotyrosine-containing proteins, such as glycoprotein Ibα

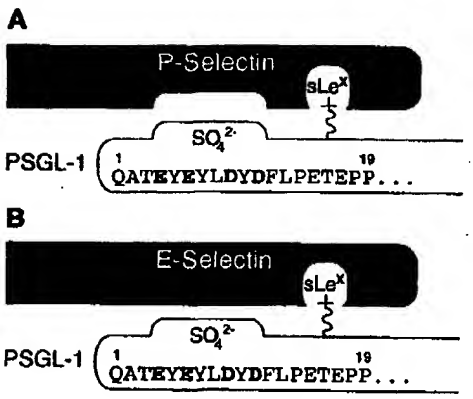

Figure 6. The Sulfated Peptide Segment in PSGL-1 Is Critical for P-Selectin Binding, but Not E-Selectin Binding

The amino-terminal 19 amino acids of mature (PACE-cleaved) PSGL-1 are shown. A bulge over residues 4–11 denotes one recognition component, comprising one or more sulfated tyrosines and four acidic residues. The structure drawn above position 16 indicates the second component, an O-linked oligosaccharide modified with sLe^x or possibly another sialylated fucosylated structure. Both components are necessary for high affinity binding to P-selectin, but only the latter component is critical for E-selectin binding.

(Dong et al., 1994), hirudin (Rydel et al., 1990), and factor VIII (Leyte et al., 1991), yet no direct homology of residues 1–19 of PSGL-1 with any other protein has been found in the GenBank or Swissprot protein database. Each of the above proteins is involved in a ligand- or receptor-binding interaction and requires tyrosine sulfation for full activity. It is interesting to note that the mouse homolog of PSGL-1 also contains a consensus site for tyrosine sulfation at the analogous amino-terminal position, as well as several acidic residues (Yang et al., submitted). Electrostatic binding interactions mediated by the sulfated region of PSGL-1 are consistent with the recently measured high tensile strength of the unimolecular interaction between purified P-selectin and PSGL-1 on neutrophils (Alon et al., 1995). The exact position and number of sulfation sites, as well as the precise structure of the fucosylated oligosaccharide at Thr-16, are the subject of ongoing studies.

The location of the site on P-selectin that interacts with the anionic, sulfated amino-terminal region of PSGL-1 remains to be determined. Several mutagenesis studies suggest that a common site exists on P- and E-selectin for calcium-dependent carbohydrate binding (Erbe et al., 1992, 1993; Hollenbaugh et al., 1993; Bajorath et al., 1994). Crystallographic studies on E-selectin (Graves et al., 1994) indicate that the binding site for sLe^x within the lectin domain is very small. Given this data, it seems unlikely that the binding site on P-selectin for the sulfated peptide closely overlaps the sLe^x binding site. Two reasonable alternatives are, first, a region outside of the sLe^x-binding site but still within the P-selectin lectin domain and, second, the P-selectin epidermal growth factor-like domain. The latter possibility is supported by studies indicating the P-selectin epidermal growth factor domain to be

critical for high affinity binding (Kansas et al., 1994; Gibson et al., 1995) and by the fact that this domain is highly conserved among species. Additional studies are required to define fully the binding sites on P-selectin and resolve this issue.

Lastly, it is interesting to consider the macromolecular structure of PSGL-1 in terms of its physiological function. Experiments using immunogold electron microscopy have indicated that the surface distribution of PSGL-1 on neutrophils is localized at the tips of microvilli (Moore et al., 1995). Our data further localize the critical binding sites to the amino-terminal segment of PSGL-1. The inclusion of the segment from amino acid residues 48–253 did not appreciably enhance the P-selectin-binding properties of our soluble PSGL-1 chimeras. Yet this segment of PSGL-1, which includes the 15 decameric repeats, contains the highest density of O-linked oligosaccharides. Mucin domains having closely packed O-linked carbohydrates are postulated to form rigid rod-like structures extending outward from the cell surface (Jentoff, 1990). It is therefore likely that the high density O-linked segment in PSGL-1 serves to extend the amino-terminal binding determinants outward from the cell surface, where they are readily accessible for selectin binding. In this regard, a rapid association rate constant for P-selectin has been noted (Lawrence and Springer, 1991; Ushiyama et al., 1993). Thus, it is logical that the binding determinants are localized at the amino terminus. Moreover, at physiological shear stress, this configuration is likely to facilitate the efficient tethering and rolling of neutrophils along the blood vessel wall.

Experimental Procedures

Plasmid Constructions

The plasmid pED.253.Fc, expressing a P-selectin ligand protein–IgG chimera, was generated by restricting plasmid pPL85 (Sako et al., 1993) with XbaI and HincII and ligating the purified 950 bp fragment to vector pED.Fc. pED.Fc is expression vector pED (Kaufman et al., 1991) having a cDNA insert encoding the Fc segment of human IgG1 with an adapter sequence immediately 5′ to the hinge region. The fusion of the extracellular portion of PSGL-1 after the valine codon 295 encodes amino acids LRPQSR DKTHTCPPC at the junction. Plasmid constructs pED.148.Fc, pED.47.Fc, and pED.19.Fc were created by standard PCR technique, using pED.253.Fc as template and the following pairs of oligonucleotide primers. Universal upstream primer: 5′-CCAGGTCCAACTGCAGGTCGACTCTAGAGGGCACTTCTTCTGGGCCCACG. Downstream primer for 148.Fc: 5′-TATTATCTGTGCGGCCGCCCTCCAGAACCCATGGCTGCTGGTTGCAGTGG. Downstream primer for 47.Fc: 5′-TATTATCTGTGCGGCCGCGCAGCAGGCTCCACAGTGGTAG. Downstream primer for 19.Fc: 5′-TATTATCTGTGCGGCCGCGGAGGCTCCGTTTCTGGCAG. PCR product DNA was digested with PstI and NotI, gel purified, and ligated with pED.Fc vector fragment. Correct constructs were identified by restriction analysis and confirmed by DNA sequencing. Plasmids pED-.ΔY148.Fc, H24Q70.148.Fc, and A16.19.Fc were created by site-directed mutagenesis (Sambrook et al., 1989) using pED.148.Fc or pED.19.Fc as templates. The mutagenesis oligonucleotide for ΔY148 was 5′-CGGAGACAGGCCACCGAATTCCTGCCAGAAACG. Positive colonies were identified by colony hybridization (Sambrook et al., 1989). The pED.FFFE.148.FC vector was constructed by restricting pED.ΔY148.Fc with EcoRI and ligating the following annealed oligonucleotides: 5′-AATTCGAGTTCCTAGATTTTG and 5′-AATTCAAAATCTAGGAACTCG. The pED.FFF.19.Fc vector was made by restricting pED.ΔY148.Fc with EcoRI and NotI and ligating the following annealed oligonucleotides: 5′-AATTCGAGTTCCTAGATTTCGATTTCCTGCCAGAAACTGAGCCTCCGC and 5′-GGCCGCGGAGGCTCAGTTTCTGGCAGGAAATCGAAATCTAGGAACTCG.

Production and Purification of Selectin Chimeras

P-selectin cDNA was fused after the encoded fourth consensus repeat domain to IgG1 Fc to create a soluble P-selectin–IgG chimera in vector pED. The resulting expression plasmid was used to establish a stable CHO cell line. Secreted chimeras were purified from serum-free medium by conventional chromatography. E-selectin–IgG chimera was constructed by fusing coding sequences of complete extracellular domain of E-selectin with human IgG1 Fc of pED.Fc. An AscI fragment containing the fusion cDNA was ligated at polylinker of baculovirus vector pVL1393 (Webb and Summers, 1990), creating plasmid pVLsE.Fc. Recombinant baculovirus was generated by cotransfecting Sf9 cells with 3 μg of pVLsE.Fc plasmid DNA, 1 μg of BaculoGold AcMNPV viral DNA (Pharmingen), followed by plaque purification. High titer virus (108 pfu/ml) was used to infect Trichoplusia ni High Five cells (Invitrogen). The supernatant 72 hr postinfection was collected, and E-selectin–IgG chimera was affinity purified using protein A–Sepharose (Pierce).

Affinity Capture Assays

The E-selectin chimera, P-selectin chimera, and human IgG1 (Sigma) were covalently coupled to a gel resin via imide ester–amide exchange chemistry (Affi-Gel 15; Bio-Rad) using the protocols of the manufacturer. Plasmid DNAs encoding the various forms of sPSGL-1–Fc chimeras were cotransfected with vectors pEA.3/4FT and pEA.sPACE into COS cells using the DEAE–dextran technique (Keown et al., 1990) and labeled metabolically with [^{35}S]methionine. For sulfate incorporation experiments, cells were starved for 1 hr in sulfate-free EMEM medium (Specialty Media), supplemented with 10% of normal concentrations of cysteine and methionine (Gibco) as well as 100 mM sodium chlorate (Fluka). Labeling was carried out for 6–7 hr by adding the same medium, supplemented with 5% dialyzed fetal calf serum, 10 mM HEPES (pH 7.2), and either ^{35}S-labeled sulfate or methionine (New England Nuclear). Control labelings were done in chlorate-free medium. Conditioned media were collected after labeling, aprotinin and sodium azide were added to 0.1% and 0.05%, respectively, and the conditioned media then were centrifuged to remove cell debris and detached cells. Labeled conditioned media, mixed 1:2 with affinity capture buffer (TBS, 5 mg/ml BSA, 2 mM $CaCl_2$, 0.04% Triton X-100, and 0.02% sodium azide), were added to affinity resins, and the mixtures were gently inverted overnight at 4°C. Resins then were washed extensively with BSA-free affinity capture buffer and batch-eluted with TBS containing 5 mM EDTA and 5 mM EGTA.

For control affinity precipitations, protein A–Sepharose 4 fast flow (Pharmacia) or a polyclonal rabbit anti-PSGL-1 antibody, Rb3443, coupled to Affi-Gel 10 was used. The antibody was raised against a peptide comprising the first 15 amino acids of the mature PSGL-1 amino terminus. Quantitative analysis was carried out by using ImageQuaNT software and a phosphorimager (Molecular Dynamics). Data were normalized for differential expression and labeling by using Rb3443 immunoprecipitation data.

Cell Binding Assay

COS cells were transfected with the various PSGL-1–IgG chimera plasmids as for the affinity capture assay. Serum-free medium (50 ml), collected 40–64 hr posttransfection from 1 × 10^7 COS cells, was purified on a column of 0.25 ml of protein A–Sepharose equilibrated with TBS plus 2 mM $CaCl_2$. After washing with 20 ml of TBS plus 2 mM $CaCl_2$, the bound material was eluted with 0.5 ml of 0.1 M acetic acid, 0.15 M NaCl, 2 mM $CaCl_2$. The eluted material was neutralized with 5% volume of 3 M Tris (pH 9.0) and dialyzed against TBS plus 2 mM $CaCl_2$, 0.1 mM PMSF. The material was quantitated by measuring absorbance at 280 nm and by Coomassie blue staining of SDS–polyacrylamide–Laemmli gels. The protein concentration was adjusted to 2 μg/ml prior to assay.

Microtiter plates (48 well, untreated; Corning) were coated for 16 hr at 4°C with 0.2 ml of purified sPSGL-1–Fc chimeras. The unbound material was removed, and the coated wells were treated with Hanks' buffered salt solution with 10 mg/ml BSA and 2 mM $CaCl_2$ for 1 hr at 4°C. CHO–P-selectin, CHO–E-selectin, and CHO–DUKX cells (Larsen

et al., 1992) were labeled by overnight incubation with ^{3}H-thymidine (New England Nuclear), and counts per minute per number of cells was recorded. Cells (2×10^5) were added to the prepared microtiter plates and incubated for 8 min at room temperature. The plates were washed four times with Hanks' buffered salt solution, 2 mM $CaCl_2$ and trypsinized, and the bound cells were quantitated by scintillation counting.

Sialidase Treatment of PSGL-1 Chimeras

[^{35}S]methionine-labeled 253.Fc or 19.Fc mutant constructs, purified from conditioned medium by protein A–Sepharose chromatography, were incubated with sialidase from Arthrobacter ureafaciens (Calbiochem) in a buffer containing 150 mM NaCl, 60 mM sodium acetate, 8 mM calcium chloride, and 0.02% Triton X-100 at pH 5.5. After a 16 hr digestion with 200 mU/ml enzyme at 37°C, the samples were diluted into affinity capture buffer, incubated with either P-selectin coupled to Affi-Gel 15 or rabbit polyclonal antibody Rb3443 coupled to Affi-Gel 10, and processed as described above.

Alkaline Hydrolysis Treatment of 19.Fc

Transfected COS cells were labeled with [^{35}S]sulfate as above, and the conditioned medium was subjected to barium hydroxide hydrolysis as described by Huttner (1984). The following modifications were performed as described previously (Pittman et al., 1992). The ^{35}S-labeled conditioned medium was incubated with protein A–Sepharose, and the bound protein was run on a 12% SDS–polyacrylamide gel and transferred to Immobilon-P (Millipore). The filter was exposed to film, and the labeled band of interest was excised from the membrane and transferred to a Tuftainer (Pierce). Degassed 0.2 M barium hydroxide was added, and the tube was sealed under N_2 and baked for 18 hr at 110°C. Following neutralization and lyophilization, the pellet was resuspended in 7.8% acetic acid, and 2.2% formic acid. We added 5 μg each of unlabeled tyrosine sulfate and threonine sulfate to the sample to run as standards. The sample was spotted onto 20 × 20 cm 100 μm cellulose thin-layer sheets (Kodak) and two-dimensional electrophoresis was performed. The standards were visualized with 1% ninhydrin in acetone, and tyrosine [^{35}S]sulfate was detected by phosphorimager analysis (Fuji) and autoradiography.

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References

Alon, R., Hammer, D.A., and Springer, T.A. (1995). Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. Nature *374*, 539–542.

Asa, D., Raycroft, L., Ma, L., Aeed, P.A., Kaytes, P.S., Elhammer, A.P., and Geng, J.G. (1995). The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. J. Biol. Chem. *270*, 11662–11670.

Bajorath, J., Hollenbaugh, D., King, G., Harte, W., Eustice, D.C., Darveau, R.P., and Aruffo, A. (1994). Cd62/P-selectin binding sites for myeloid cells and sulfatides are overlapping. Biochemistry *33*, 1332–1339.

Baumhueter, S., Singer, M.S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S.D., and Lasky, L.A. (1993). Binding of L-selectin to the vascular sialomucin CD34. Science *262*, 436–438.

Berg, E.L., Mcevoy, L.M., Berlin, C., Bargatze, R.F., and Butcher, E.C. (1993). L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature *366*, 695–698.

Brandley, B.K., Kiso, M., Abbas, S., Nikrad, P., Srivasatava, O., Foxall, C., Oda, Y., and Hasegawa, A. (1993). Structure-function studies on selectin carbohydrate ligands: modifications to fucose, sialic acid and sulphate as a sialic acid replacement. Glycobiology *3*, 633–641.

Capon, D., Charmow, S., Mordenti, J., Marsters, S., Gregory, T., Mitsuya, H., Byrn, R., Lucas, C., Wurm, F., Groopman, G., Broder, S., and Smith, D. (1989). Designing CD4 immunoadhesins for AIDS therapy. Nature *337*, 525–531.

Dong, J., Li, C.Q., and Lopez, J.A. (1994). Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding. Biochemistry *33*, 13946–13953.

Erbe, D.V., Wolitzky, B.A., Presta, L.G., Norton, C.R., Ramos, R.J., Burns, D.K., Rumberger, J.M., Rao, B.N.N., Foxall, C., Brandley, B.K., and Lasky, L.A. (1992). Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. J. Cell Biol. *119*, 215–227.

Erbe, D.V., Watson, S.R., Presta, L.G., Wolitzky, B.A., Foxall, C., Brandley, B.K., and Lasky, L.A. (1993). P- and E-selectin use common sites for carbohydrate ligand recognition and cell adhesion. J. Cell Biol. *120*, 1227–1235.

Foxall, C., Watson, S.R., Dowbenko, D., Fennie, C., Lasky, L.A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B.K. (1992). The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis X oligosaccharide. J. Cell Biol. *117*, 895–902.

Gibson, R.M., Kansas, G.S., Tedder, T.F., Furie, B., and Furie, B.C. (1995). Lectin and epidermal growth factor domains of P-selectin at physiologic density are the recognition unit for leukocyte binding. Blood *85*, 151–158.

Graves, B.J., Crowther, R.L., Chandran, C., Rumberger, J.M., Li, S., Huang, K.S., Presky, D.H., Familletti, P.C., Wolitzky, B.A., and Burns, D.K. (1994). Insight into E-selectin ligand interaction from the crystal structure and mutagenesis of the lec/EGF domains. Nature *367*, 532–538.

Hemmerich, S., and Rosen, S.D. (1994). 6'-Sulfated sialyl Lewis X is a major capping group of Glycam-1. Biochemistry *33*, 4830–4835.

Hemmerich, S., Bertozzi, C.R., Leffler, H., and Rosen, S.D. (1994). Identification of the sulfated monosaccharides of Glycam-1, an endothelial-derived ligand for L-selectin. Biochemistry *33*, 4820–4829.

Hemmerich, S., Leffler, H., and Rosen, S.D. (1995). Structure of the O-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. J. Biol. Chem. *270*, 12035–12047.

Hollenbaugh, D., Bajorath, J., Stenkamp, R., and Aruffo, A. (1993). Interaction of P-selectin (CD62) and its cellular ligand: analysis of critical residues. Biochemistry *32*, 2960–2966.

Huttner, W.B. (1984). Determination and occurrence of tyrosine O-sulfate in proteins. Meth. Enzymol. *107*, 200–223.

Huttner, W.B., and Baeuerle, P.A. (1988). Protein sulfation on tyrosine. Mod. Cell Biol. *6*, 97–140.

Imai, Y., Lasky, L.A., and Rosen, S.D. (1993). Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. Nature *362*, 555–557.

Jentoft, N. (1990). Why are proteins O-glycosylated? Trends Biochem. Sci. *15*, 291–294.

Kansas, G.S., Saunders, K.B., Ley, K., Zakrzewicz, A., Gibson, R.M., Furie, B.C., Furie, B., and Tedder, T.F. (1994). A role for the epidermal growth factor-like domain of P-selectin in ligand recognition and cell adhesion. J. Cell Biol. *124*, 609–618.

Kaufman, R.J., Davies, M.V., Wasley, L.C., and Michnick, D. (1991). Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. Nucl. Acids Res. *19*, 4485–4490.

Keown, W.K., Campbell, C.R., and Kucherlapati, R.S. (1990). Methods for introducing DNA into mammalian cells. In Methods in Enzymology: Gene Expression Technology, D.V. Goeddel, ed. (Orlando, Florida: Academic Press Incorporated), pp. 527–537.

Larsen, G.R., Sako, D., Ahern, T.J., Shaffer, M., Erban, J.E., Sajer, S.A., Gibson, R.M., Wagner, D.D., Furie, B.C., and Furie, B. (1992). P-selectin and E-selectin: distinct but overlapping leukocyte ligand specificities. J. Biol. Chem. *267*, 11104–11110.

Lasky, L.A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science *258*, 964–969.

Laskey, L.A., Singer, M.S., Dowbenko, D., Imai, Y., Henzel, W.J., Grimley, C., Fennie, C., Gillett, N., Watson, S.R., and Rosen, S.D.

(1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell *69*, 927–938.

Lawrence, M.B., and Springer, T.A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859–873.

Leyte, A., van Schijndel, H.B., Niehrs, C., Huttner, W.B., Verbeet, M.P., Mertens, K., and van Mourik, J.A. (1991). Sulfation of Tyr^{1680} of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. J. Biol. Chem. *266*, 740–746.

Lipmann, F. (1958). Biological sulfate activation and transfer. Science *128*, 575–580.

Lowe, J.B., Stoolman, L.M., Nair, R.P., Larsen, R.D., Berhend, T.L., and Marks, R.M. (1990). ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyl-transferase cDNA. Cell *63*, 475–484.

Moore, K.L., Stults, N.L., Diaz, S., Smith, D.F., Cummings, R.D., Varki, A., and McEver, R.P. (1992). Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J. Cell Biol. *118*, 445–456.

Moore, K.L., Eaton, S.F., Lyons, D.E., Lichenstein, H.S., Cummings, R.D., and Mcever, R.P. (1994). The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetyllactosamine. J. Biol. Chem. *269*, 23318–23327.

Moore, K.L., Patel, K.D., Bruehl, R.E., Fugang, L., Johnson, D.A., Lichenstein, H.S., Cummings, R.D., Bainton, D.F., and McEver, R.P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J. Cell Biol. *128*, 661–671.

Natsuka, S., Gersten, K.M., Zenita, K., Kannagi, R., and Lowe, J.B. (1994). Molecular cloning of a cDNA encoding a novel human leukocyte α-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. J. Biol. Chem. *269*, 16789–16794.

Norgard, K.E., Moore, K.L., Diaz, S., Stults, N.L., Ushiyama, S., Mcever, R.P., Cummings, R.D., and Varki, A. (1993). Characterization of a specific ligand for P-selectin on myeloid cells: a minor glycoprotein with sialylated O-linked oligosaccharides. J. Biol. Chem. *268*, 22953.

Pittman, D.D., Wang, J.H., and Kaufman, R.J. (1992). Identification and functional importance of tyrosine sulfate residues within recombinant factor VIII. Biochemistry *31*, 3315–3325.

Rehemtulla, A., and Kaufman, R.J. (1992). Protein processing within the secretory pathway. Curr. Opin. Biotech. *3*, 560–565.

Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J.W., II (1990). The structure of a complex of recombinant hirudin and human α-thrombin. Science *249*, 277–280.

Sako, D., Chang, X.-J., Barone, K.M., Vachino, G., White, H.M., Shaw, G., Veldman, G.M., Bean, K.M., Ahern, T.J., Furie, B., Cumming, D.A., and Larsen, G.R. (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. Cell *75*, 1179–1186.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Press).

Sasaki, K., Kurata, K., Funayama, K., Nagata, M., Watanabe, E., Ohta, S., Hanai, N., and Nishi, T. (1994). Expression cloning of a novel α1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis x carbohydrate determinants in leukocytes. J. Biol. Chem. *269*, 14730–14737.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301–314.

Ushiyama, S., Laue, T.M., Moore, K.L., Erickson, H.P., and McEver, R.P. (1993). Structural and functional characterization of monomeric soluble P-selectin and comparison with membrane P-selectin. J. Biol. Chem. *268*, 15229–15237.

Vachino, G., Chang, X.-J., Veldman, G.M., Kumar, R., Sako, D., Fouser, L.A., Berndt, M.C., and Cumming, D.A. (1995). P-selectin glycoprotein ligand-1 is the major counter-receptor for P-selectin on stimulated T cells and is widely distributed in non-functional form on many lymphocytic cells. J. Biol. Chem. *270*, 21966–21974.

Varki, A. (1994). Selectin ligands. Proc. Natl. Acad. Sci. USA *91*, 7390–7397.

Webb, N.R., and Summers, M.D. (1990). Expression of proteins using recombinant baculoviruses. Technique *2*, 173–188.

Wilson, I.B.H., Gavel, Y., and von Heijne, G. (1991). Amino acid distributions around O-linked glycosylation sites. Biochem. J. *275*, 529–534.

Yuen, C.-T., Lawson, A.M., Chai, W., Larkin, M., Stoll, M.S., Stuart, A.C., Sullivan, F.X., Ahern, T.J., and Feizi, T. (1992). Novel sulfated ligands for the cell adhesion molecule E-selectin revealed by the neoglycolipid technology among O-linked oligosaccharides on an ovarian cystadenoma glycoprotein. Biochemistry *31*, 9126–9131.

Zhou, Q., Moore, K.L., Smith, D.F., Varki, A., McEver, R.P., and Cummings, R.D. (1991). The selectin GMP-140 binds to sialylated, fucosylated lactosaminoglycans on both myeloid and nonmyeloid cells. J. Cell Biol. *115*, 557–564.